Prediction of Response in Cancer Therapy

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DECEMBER 1971

Prediction of Response in Cancer Therapy

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PUBLIC HEALTH SERVICE
NATIONAL INSTITUTES OF HEALTH
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PREDICTION OF RESPONSE
IN CANCER THERAPY

Symposium held February 19–21, 1970

Cascades Conference Center

Williamsburg, Virginia

Sponsored by
Cancer Clinical Investigation
Review Committee
National Cancer Institute
National Institutes of Health
Bethesda, Maryland
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INTRODUCTION

This volume reports on the proceedings of a Conference, "Prediction of Response to Cancer Therapy." The Conference was held in Williamsburg, Virginia, February 19–21, 1970. It was sponsored by the Cancer Clinical Investigation Review Committee (CCIRC) of Extramural Activities of the National Cancer Institute (NCI). The CCIRC is charged with an overview of cooperative studies and clinical investigations outside of the intramural programs of the NCI. These activities represent the major interaction between the NCI and the community, as represented by the physician and his patients.

The treatment of cancer has become more effective. Surgical therapy can cure about 33% of all malignancies in the stages in which they present for the first time. Since this percentage has not changed recently, one must assume that unnecessary surgery is performed two-thirds of the time. Much morbidity and economic loss could be avoided if we could select for surgery only those patients who could be cured. Factors which might permit such preselection are: detailed study of the staging of the tumor, the morphology of tumor nuclei, the capacity of the tumor to invade blood vessels, as well as the local nodal and generalized immunologic reactivity of the host.

In radiation therapy, staging, study of tumor histology, host tissue vascularity, and cytology have been proposed as factors that could be sampled before treatment to predict the radio-responsiveness or radio-resistance of specific tumors.

The intramural Drug Development Program of the NCI concentrates on new drugs of potential ultimate usefulness, and it is staffed by researchers with special capabilities for conducting new drug studies. However, most Clinical Cooperative Groups supported by the NCI use more conventional drugs and treatments, such as surgery and radiation. They frequently attempt to combine these agents or methods into more effective tools for treatment of patients.

Some of the existing drugs are very effective, such as the DNA antimetabolite 5-fluorouracil, as compared with others, *i.e.*, as the similar but ineffective DNA antimetabolite 6-mercaptopurine. Why is this? Some common neoplastic diseases, such as cancer of the breast, respond to multiple single therapies, such as surgical removal of endocrine glands, X irradiation, and administration of sex hormones, antimetabolites, alkylating agents, and vinca alkaloids. Others, to wit, epidermal cancer of the lung, rarely respond to treatments of any type. Why is this? A single agent, for example, methotrexate or cytosine arabinoside, will cause remissions in 40% of patients with acute lymphatic leukemia but will have little effect in the other 60%. If one could predict which patients would respond, 60% of patients could be spared unnecessary toxicity.

In chemotherapy, almost 30% of the patients can be given some palliation; plasma half-times of drugs, the protein binding of drugs, the ability of host organs to activate, destroy, or excrete drugs, and the rate of tumor cell division and cellular enzymatic apparatus all have been shown to be factors in determining those drugs or drug combinations that would be useful in selecting which patients can be helped by chemotherapy and which can only be intoxicated by chemotherapy.

If one could identify the patients who would not be cured by surgery, radiation and chemotherapy could be started earlier. If patients resistant to standard chemotherapy agents could be preselected, early studies on new drugs could be commenced in these patients. The 30–50% of the patients who respond to cancer therapies generally respond well. Understanding why some patients respond and others do not may also help us to understand why all cancers have escaped from the internal homeostatic controls to which the rest of the body's cells respond.

This Conference was organized to delineate areas of potential clinical help to the physicians in the Cooperative Groups, with a view to creating a better fit of currently available therapies to specific patients and to identify patients in whom early experimentation is justified. We owe a debt of gratitude to Dr. Laurance V. Foye, former Executive Secretary of the CCIRC, and Dr. J. Palmer Saunders, Associate Director for Extramural Affairs of the NCI, for their help in making this Symposium possible.

Thomas C. Hall, M.D.

PANEL I

CELL KINETICS AND THERAPEUTIC RESPONSE

Kinetic Behavior Versus Response to Chemotherapy[1, 2, 3]

HOWARD E. SKIPPER,[4] *Southern Research Institute, Birmingham, Alabama 35205*

AS FAR as I know, the growth rate of all tumors becomes progressively slower as they increase in mass. If a primary tumor reached a steady state at some tolerated mass in some "noncritical site" and the cells from the tumor did not metastasize and begin growth in some "critical site(s)," I assume that the tumor would not be life threatening. Various deviations from this situation characterize some of our major problems. A major limitation to surgery and radiotherapy appears to be related to widespread dissemination at diagnosis. A major limitation to chemotherapy seems to be related to the fraction of *viable* tumor cells engaged in a critical phase of DNA replication at a moment in time, *e.g.,* in the primary or metastatic foci if the primary tumor is removed.

Progressive slowing of the growth rate of tumors with increase in mass may reflect: *a*) increasing median ${}^{T}C$, sometimes with relatively less increase in median ${}^{T}S$, *b*) a decrease in the "DNA-replicating" fraction with increasing median and extreme ${}^{T}G_1 + {}^{T}M + {}^{T}G_2$ in the still viable cells, and *c*) increasing cell death, lysis, and resorption (*1–3*). These changes may occur concurrently. I suspect that these changes are associated phenomena. More specifically, an increase in ${}^{T}C$ without a comparable increase in ${}^{T}S$ implies increasing average (or fractional) sequestration in $G_1 + M + G_2$. Since increases in ${}^{T}G_1$ are accompanied by increases in tumor cell loss, is it not largely the tumor cells, which sequester longer and longer without completing DNA replication, that lose proliferative integrity, die, and are resorbed?

It seems fair to state that effective blood concentrations of some of our chemotherapeutic agents kill cells because of their effects on DNA metabolism, and as cells in nonsynchronous populations attempt to replicate their DNA.

In this study, we examined some kinetic and chemotherapeutic response data on neoplasms for which both types of data are available. Pertinent questions are:

a) Can one predict (within limits) the response of a type of cancer to chemotherapy from knowledge of kinetic behavior?

b) Which kinetic parameters (if any) correlate with the response of tumors to antimetabolites or agents which react with or complex with DNA?

c) If any such correlations exist, what are the implications with respect to therapeutic design aimed at stepwise improvement in control of more extensive types of *disseminated* cancer by chemotherapy, or chemotherapy after surgery or ionizing radiation?

KINETIC BEHAVIOR VERSUS RESPONSE TO CHEMOTHERAPY IN VARIOUS EXPERIMENTAL TUMOR SYSTEMS

Text-figure 1 and table 1 show growth rate data on a number of different experimental neoplasms. Table 2 gives a *drastic* summary of some kinetic parameters and the response to chemotherapy of these neoplasms in their early and more advanced stages. It is not easy (or satisfying) to summarize the masses of data underlying table 2 in a single simple table; the basic data may be found in (*4–26*). Long study of the raw data underlying table 2 leads to the following conclusions:

1) The doubling times of all these tumors increase with increase in tumor mass; the rates of increase in doubling time vary.

2) A consistent and unmistakable inverse relationship exists between advancement of

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[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

[3] The work from the Kettering-Meyer Laboratory of Southern Research Institute referred to in this paper was supported by Public Health Service contracts PH-43-65-594 and PH-43-66-29 (chemotherapy) from the National Cancer Institute, by grant T-111J from the American Cancer Society, Inc., and grants from the C. F. Kettering Foundation and the Alfred P. Sloan Foundation.

[4] I am particularly indebted to Drs. F. M. Schabel, Jr., D. P. Griswold, Jr., W. R. Laster, Jr., H. H. Lloyd, and L. B. Mellett and to Mrs. Linda Simpson-Herren.

TABLE 1.—Growth rate versus tumor cell number or tumor mass for experimental neoplasms for which both kinetic and therapeutic response data are available

Reference to growth curves in text-figure 1	Tumor	Induced by	Site of tumor cell population measured	Average doubling time* (days) at:		
				10^6–10^7 cells	10^8 cells	10^9 cells
A	L1210 leukemia	10^6; intraperitoneal (ip)	Disseminated	0.42	0.5 or >	Dead
B	L1210 leukemia or long-passage AK leukemia	10^6; intravenous (iv)	Disseminated	0.40	0.4 or >	Dead
C	Ehrlich ascites carcinoma	10^6; ip	ip only	0.46	1.6	5
D	Solid L1210 leukemia	10^6; subcutaneous (sc)	sc only	(<0.5)	0.5	1.7
E	Solid Dunning leukemia	10^4–10^7; sc	Disseminated	0.54		
F	First-passage AK leukemia	10^6; iv	Disseminated	ca. 1.1	>1.1	Dead
G	Advanced spontaneous AK leukemia (diagnosis to death)	Spontaneous	Disseminated			ca. ≥5
				Early, 0.01 g	0.1 g	Advanced, 1.0 g
H	Sarcoma 180	25-mg fragment, sc	sc only	(0.58)	0.77	1.9
I	Carcinoma 755	25-mg fragment, sc	sc only	(0.6)	0.95	3.2
J	Walker carcinoma 256	25-mg fragment, sc	sc only	(0.5)	0.66	1.0
K	Plasmacytoma-1	25-mg fragment, sc	sc only	(0.94)	1.2	2.4
L	Lewis lung carcinoma	25-mg fragment, sc	sc only	(1.2)	2.0	3–4
M	Solid Ehrlich carcinoma	25-mg fragment, sc	sc only	(1.4)	2.3	4.4
N	Melanotic melanoma	25-mg fragment, sc	sc only	(1.5)	2.3	3.5
O	First-passage mammary tumors	25-mg fragment, sc	sc only	(2.3)	3.3	6.7
P	7,12-Dimethylbenz[*a*]anthracene (DMBA)-induced carcinoma of breast	DMBA (not transplanted)	Mammary tumor only	(2.8)	4.6	12

* Doubling times in large numbers of animals; values in *parentheses* were back-extrapolated along a best fit Gompertzian curve for one decade below the limit of measurement.

Table 2.—Some kinetic parameters versus response to chemotherapy*

Reference to growth curves in text-figure 1	Host	Tumor	Kinetic parameters at degree of tumor advancement indicated						Response to chemotherapy†		
			Tumor cell number or mass (mg)	Doubling time (days)	Thymidine index (%)		Median cell cycle characteristics (hours)		Agents killing cells in S phase	Alkylating agents	Combinations
					Pulse	24 hours	${}^{T}S$	${}^{T}G_1+{}^{T}M+{}^{T}G_2$			
B	Mouse	L1210 leukemia, intravenous (iv)	10^5	0.4					++	+++	+++
B	Mouse	Long-passage AK leukemia (iv)	10^6	0.4					++	++	+++
A	Mouse	L1210 leukemia, intraperitoneal (ip)	10^6	0.42	65	>99	9	3.8	++	+++	+++
C	Mouse	Ehrlich ascites carcinoma (ip)	10^7	0.46	70	>95	6	2	++	++	
D	Mouse	Solid L1210 leukemia, subcutaneous (sc)	ca. 100 mg	ca. 0.5	22	47			++	+++	
E	Rat	Solid Dunning leukemia (sc)	10^4–10^7	0.54						(+++)‡	
J	Rat	Walker carcinoma 256 (sc)	1000–3000	ca. 1.0	21	26				(++ to +++)‡	
	Rat	Walker carcinoma 256 (pulmonary)								(++)‡	
F	Mouse	First-passage AK leukemia (iv)	ca. 10^6	1.1					++	++	
H	Mouse	Sarcoma 180 (sc)	500	1.5	36	85	7.5	5.5	+	++	
I	Mouse	Carcinoma 755 (sc)	400	1.9	18	42	6	8	++ (6-mercaptopurine)	+	+++
K	Hamster	Plasmacytoma-1 (sc)	1000	2.4	11–14	20–31	5.8	9.2	±	+++	+++
M	Mouse	Solid Ehrlich carcinoma (sc)	175	2.4	13–20	18	8.8	10.2	±	++	
L	Mouse	Lewis lung carcinoma (sc)	400	2.5	15	12–22	8.5	10.3	—	+++	
		Lewis lung carcinoma (pulmonary)								++ to +++	
N	Hamster	Melanotic melanoma (sc)	ca. 1000	3.5	7–12	27	6.3	11.2	—	+	
O	Mouse	First-passage mammary tumors (sc)	360–1000	4.5	ca. 1–2		9	8.8	—	+	
G	Mouse	Advanced spontaneous AK leukemia (diagnosis to death)	ca. 10^9	⩾5	5–37		ca. 6	ca. 14	—	+	(+ to +++?)‡§
P	Rat	7,12-Dimethylbenz[*a*]-anthracene (DMBA)-induced carcinoma of breast	1000–3000	12–20	10		8.5	13.5	—	±	

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* *See* (4–26).

† +++ = "cure" of advanced metastic disease; ++ = "cure" of early disease (usually metastatic); + = significant increase in host survival time or marked tumor mass regression, or complete inhibition of early tumor growth during treatment; ± = moderate-to-marked inhibition of tumor growth; and — = slight tumor inhibition or no observable effects. These ratings are all from confirmed results (mostly very extensive); however, they generally represent response to the most effective agent in a class (or combination) at optimal or near-optimal schedules. By now, it is well established that optimal scheduling often may make the difference between success and failure in treating various types of disseminated animal cancer.

‡ Ratings in *parentheses* represent earlier work in which animals were not observed long enough to be certain that recurrences would not occur.

§ About 15% of the animals survived for longer than 2 months after the cessation of treatment, but eventually they died of lymphoma. Bioassay data indicate that some "cell cures" may have been achieved, only to be followed by reinduction of the cellular phase of the disease in these animals which carry the Gross virus as well as a genetic susceptibility to this virus.

neoplastic disease and "curability" by chemotherapy.

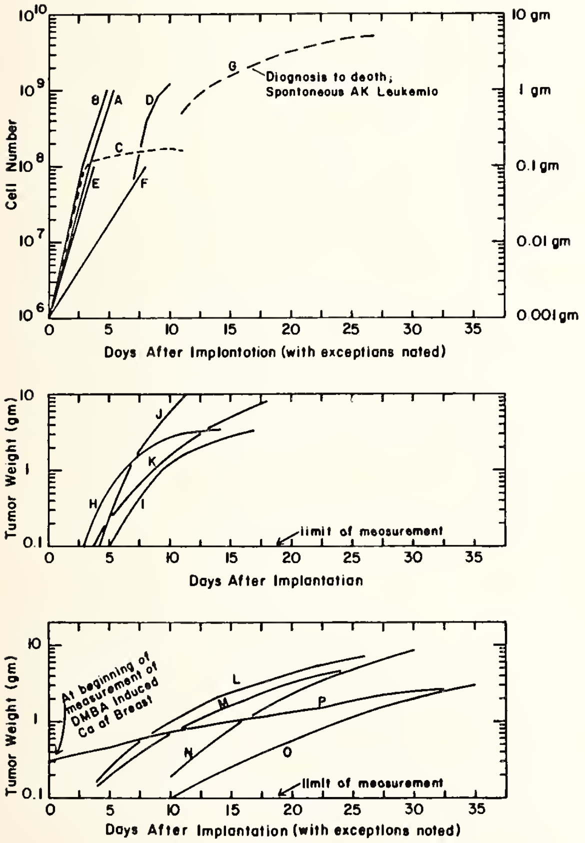
3) An inverse relationship exists between the doubling time of these tumors at initiation of treatment and response to chemotherapy.

4) A direct relationship exists between thymidine index and response to chemotherapy.

5) Apparently an inverse relationship exists between ${}^{T}G_1 + {}^{T}M + {}^{T}G_2$ and response to chemotherapy, but no obvious relationship exists between median ${}^{T}S$ of the dividing cells and "sensitivity."

6) Alkylating agents (when optimally used) generally appear more effective against tumors with longer doubling times, lower thymidine indexes, and longer ${}^{T}G_1 + {}^{T}M + {}^{T}G_2$ than the optimally used S-phase-specific drugs (*e.g.*, arabinosylcytosine). Combinations which include alkylating agents are sometimes even more effective when optimally used.

7) The odds against such relationships occurring by chance alone must be very high.



TEXT-FIGURE 1.—Growth rates of some experimental neoplasms on which chemotherapeutic response data are available. DMBA = 7,12-dimethylbenz[*a*]anthracene. Leukemias and Ehrlich ascites carcinoma; all in the mouse excepting Dunning leukemia in the rat:

A. L1210 leukemia, intraperitoneal (ip)
B. L1210 leukemia or long-passage AK leukemia, intravenous (iv)
C. Ehrlich ascites carcinoma (ip)
D. Solid L1210 leukemia, subcutaneous (sc)
E. Solid Dunning leukemia (sc)
F. First-passage AK leukemia (iv)
G. Advanced spontaneous AK leukemia (estimated)

"Rapidly growing" solid tumors:

H. Sarcoma 180 (sc); mouse
I. Carcinoma 755 (sc); mouse
J. Walker carcinoma 256 (sc); rat
K. Plasmacytoma (sc); hamster

"Less rapidly growing" solid tumors:

L. Lewis lung carcinoma (sc); mouse
M. Solid Ehrlich carcinoma (sc); mouse
N. Melanotic melanoma (sc); hamster
O. First-passage mammary tumors (sc); C3H mouse
P. DMBA-induced carcinoma of breast; rat

The above conclusions are consistent with a large body of data on the "similar sensitivity" to chemotherapy of a wide variety of animal and human tumor cell populations—after they have been "uncrowded and adapted" to log-phase culture *(27, 28)*. Such log-phase cultures of tumor cell populations usually have doubling times of about 0.5–1.5 days, pulse thymidine indexes of about 50%, and 24-hour thymidine indexes of >90%, regardless of the growth rates, thymidine indexes, or the sensitivity to chemotherapy of the original tumors from which the cell cultures were derived *(27–32)*.

In retrospect, a biochemist can rationalize much of what has now been observed on the *in vivo* response of cancer cell populations to S-phase-specific drugs with the classical pioneering work of Cohen and Barner *(33)*, *i.e.*, "unbalanced growth" in *Escherichia coli*.

KINETIC BEHAVIOR OF CERTAIN HUMAN TUMORS

Some kinetic data on human neoplasms are listed in tables 3 and 4. The response of a wide variety of human neoplasms (usually far advanced) to chemotherapy may be found in *(37, 41–44)* and elsewhere. In the light of concepts put forth by Mendelsohn *(1, 2)* and Steel and Lamerton *(3, 34)*, with respect to growth fraction and cell loss, it would be expected that many large human tumors would have long volume doubling times and low thymidine indexes. If the kinetic and therapeutic response data summarized in table 2 are compared with data in tables 3 and 4 (and basic knowledge of the cytotoxic specificity of certain anticancer agents for "dividing" cells), some light is shed on our basic problems. Such a comparison suggests *a*) why some rapidly growing animal tumor systems are reasonable models for some rapidly growing human neoplasms and *b*) by implication, that some "slowly growing" animal neoplasms might be reasonable models for some "slowly growing" human neoplasms.

Table 3.—Labeling indexes and potential doubling times of human tumors (for comparison with table 2)

Site of tumor[*]	Median pulse thymidine index (%)	Potential doubling time[†] (days)	Reference
Lymphosarcoma, Burkitt's tumor	32	1.5	(*34*)
Acute lymphoblastic leukemia (marrow), "large" cells	ca. 45	ca. 1.0	(*35*)
Acute lymphoblastic leukemia (marrow), "all" cells	ca. 5	ca. 9.4	(*36*)
Lung and larynx	15	3.1	(*34*)
Tongue	7.2	6.5	(*34*)
Stomach	6.9	6.8	(*34*)
Uterus and cervix	4.8	9.8	(*34*)
Colon	4.5	10.4	(*34*)
Melanoma	3.3	14.2	(*34*)
Central nervous system	2.0	23.4	(*34*)
Breast	1.1	43	(*34*)
Various solid animal and human tumor cell populations in log-phase culture (doubling time ca. 0.5–1.5 days)	ca. 50	ca. 1.0	(*28–31*)
Acute myelocytic leukemia[‡] in log-phase culture (doubling time ca. 1.0 day)	50	ca. 1.0	(*32*)

[*] Steel and Lamerton (*34*) reported that the distribution of 175 published measurements of the volume doubling times of various solid human tumors ranged from <10–>300 days, with a median of 60–70 days. Doubtlessly these were tumors of about 1 g to "very large." Probably disseminated Burkitt's tumor and acute lymphocytic leukemia are more responsive to chemotherapy (including S-phase-specific agents) than are the other human tumors listed (*37*). Solid tumor cell populations, when adapted to log-phase culture, appear to become about equally sensitive to chemotherapeutic agents (*27, 28*).

[†] Potential doubling time (of rather large tumors) in the absence of cell loss $= \frac{T_S}{T_I} \cdot \lambda$ (*34*). The above calculations were made with $T_S = 15$ hours. Some of these potential doubling times might be somewhat shorter in very small tumors or after tumor perturbation

[‡] Peripheral blood cells from a patient with myeloblastic leukemia.

Table 4.—Cell cycle characteristics of several human tumor cell populations (for comparison with table 2)

Tumor	Median (hours)[*] T_S	Median (hours)[*] $T_{G_1} + T_M + T_{G_2}$	Reference
Advanced primary skin tumors			
Patient 1	19	78	(*38*)
2	19	48	(*38*)
3	11	14	(*38*)
4	12	36	(*38*)
5	21	241	(*38*)
"Small" metastatic malignant melanoma in patient No.:			
Patient 1	24	—	(*39*)
2	24	58	(*39*)
3	22	—	(*39*)
Melanoma	21	ca. 72	(*40*)
"Small" metastatic carcinoma of breast in patient No.:			
Patient 1	19	—	(*39*)
2	22	—	(*39*)
3	24	18	(*39*)
Acute lymphoblastic leukemia and acute myelocytic leukemia	ca. 20	ca. 30–40	(*36*)
Human cancer cells in log-phase culture:[†]			
Acute myelocytic leukemia[‡]	12	6	(*32*)
KB (carcinoma of nasopharynx)	12	9	(*30*)
HEp 2 (carcinoma of larynx)	7	19	(*31*)
HeLa (carcinoma of cervix)	6–10	14–18	(*29*)

[*] The median, range, and coefficient of variation of $T_{G_1} + T_M + T_{G_2}$ of tumor cell populations (before and after perturbation) seem to have important implications with respect to chemotherapy.

[†] All the log-phase human cell populations are "sensitive" to chemotherapeutic agents, including S-phase-specific agents.

[‡] Peripheral blood cells from a patient with myeloblastic leukemia adapted to log-phase culture.

IMPLICATIONS WITH RESPECT TO TACTICS

A. The evidence available suggests that surgical removal or *in situ* destruction by radiation or chemotherapy of most cells in a tumor (or host) can leave a cell population(s) which, for a while, grows more rapidly (*see* table 5 and text-fig. 2). This smaller remaining population can be more sensitive to chemotherapy than were all viable cells in the original unperturbed tumor, on a fractional cell-kill-per-dose basis (*see* table 5 and text-fig. 3). A bonus is that such perturbation may have reduced the total cell number by several decades (*45*).

TABLE 5.—Tumors induced by equal-sized fragments taken from small, rapidly growing tumors and from large, slowly growing tumors appear at the same time and grow at the same rates (Carcinoma 755 system)

Donor tumors which were harvested and surgically "perturbed" (*i.e.*, 25-mg fragments were dissected for transplantation)			Average mass doubling time (days) of tumors induced by 25-mg fragments from the donor tumors on the following postimplant days					
Days post implant	Average mass (mg)	Mass doubling time at time of harvest (days)	6	8	10	12	14	16
8	220	1.2	0.9	1.3	1.8	2.7	4.1	6.7
10	650	1.8	1.5	1.8	2.3	3.4	4.2	5.3
12	1400	2.7	1.1	1.5	2.0	2.7	3.8	5.5
14	1900	4.2	1.1	1.5	2.1	2.8	4.0	5.8
16	3400	7.4	1.6	2.0	2.5	3.2	4.1	5.2
18	3900	>10	1.2	1.6	2.1	2.9	3.9	5.4
20	5200	>10	1.2	1.6	2.3	3.3	5.0	8.2
		Median....	1.2	1.6	2.1	2.9	4.1	5.5

Note: Limit of tumor measurement is about 60 mg. The viable cells in the small fragments transplanted do not "remember" the doubling time of the tumor from which they were dissected.

Kinetic parameters of Carcinoma 755 with increase in mass

Days post implant	Average mass (mg)	Mass doubling time (days)	Calculated potential doubling time (days)[a]	*TC* (hours)	*TS* (hours)	*TS*/*TC*	Pulse thymidine index (%)	Calculated proliferating fraction[c]	Response to therapy
4	46	0.77	0.89 (—)[b]	12	7	0.58	27	0.50	"Curable" by 6-mercaptopurine alone
6	76	1.29							
8	378	1.9	1.1 (27)[b]	14	6	0.43	18	0.44	"Curable" by cytoxan➔6-mercaptopurine
14	2660	>10	2.1 (546)[b]	16	6	0.37	9	0.27	
50% of animals dead.....19	3660	>10	5.3 (240)[b]	24	15.5	0.65	9	0.14	

Note: (*a*) Potential doubling time in the absence of cell loss = $TS/TI \cdot \lambda$ (*3*).

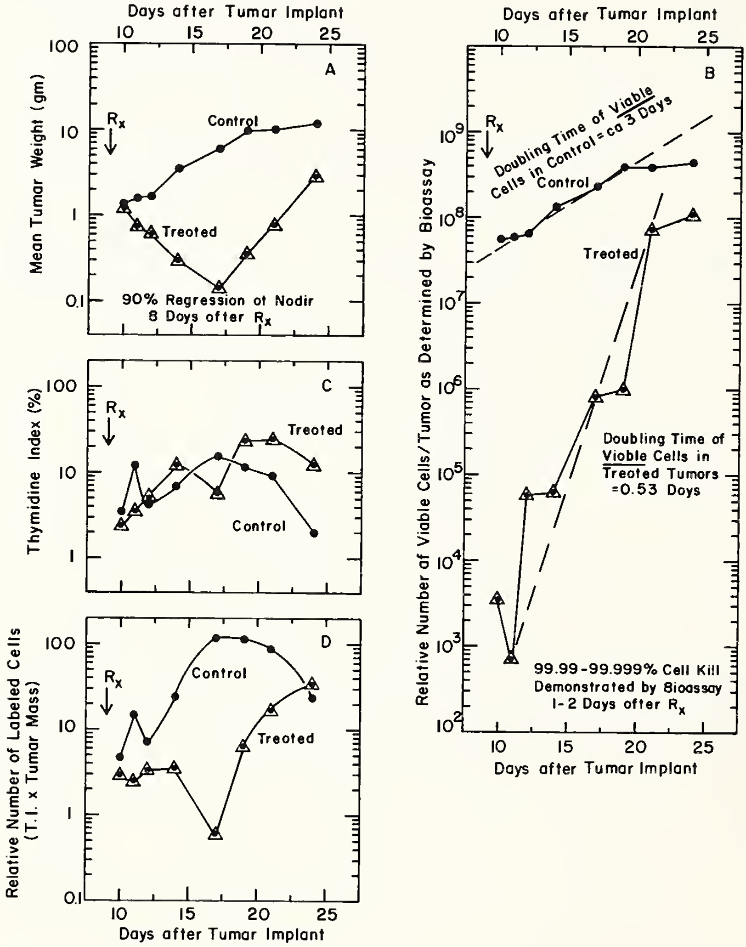
(*b*) Calculated cell loss in mg/day.

(*c*) $\frac{TI}{TS/TC \cdot \lambda}$

Conclusions: The increase in doubling time of this tumor cannot be accounted for by (*a*) increase in *TC* alone, or (*b*) increase in cell loss alone, or (*c*) decrease in proliferating fraction alone. It seems most reasonable to assume that increase in *TC*, decrease in proliferating fraction, and increase in cell loss are part and parcel of related phenomena which are reflected in asymptotic tumor growth. At the chemotherapy (or biochemical) level, it would seem that it is increasing $TG_1 + TM + TG_2$ (before cell death) which underlies our major hurdle. Blood supply, crowding, etc., may be the responsible biologic phenomena.

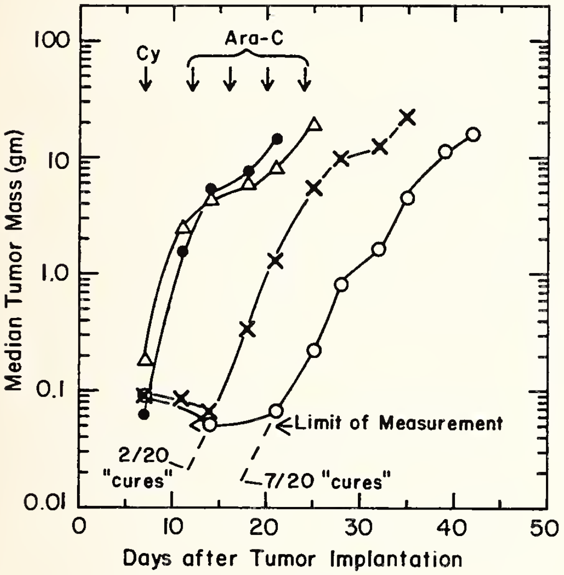
B. We are advancing stepwise in selecting agents and optimal therapeutic regimens which are "curing" some extremely disseminated animal cancers. Kinetic, biochemical, and pharmacologic information has been very helpful; however, I am in complete agreement with Bruce and Lin *(46)* that the availability of direct and indirect methods for estimating the fraction of various tumor cell populations which survive a given dose or course of treatment has been invaluable at the experimental level. Knowledge and guidance (with respect to selecting optimal therapeutic regimens) which might take years to obtain by trial and error can be gained in weeks with a satisfactory assay for "proliferative integrity," along with appropriate host-toxicity data.

Progress has been made with respect to long-term control of a few types of *disseminated* human cancer by chemotherapy, radiotherapy, or these modalities after surgery. If we are to extend such successes in a stepwise fashion to types of human cancer more difficult to cure—and wish to do this



TEXT-FIGURE 2.—Rate of increase in viable tumor cells after chemotherapeutic perturbation and the serious underestimation of tumor cell kill, based solely on tumor mass behavior (Wilcox phenomenon). These data are from a single experiment carried out by Dr. Daniel P. Griswold and Mrs. Linda Simpson-Herren. The tumor system is plasmacytoma-1 in the hamster. The animals received a single dose of Cytoxan (20 mg/kg) on day 9. A: Tumor mass behavior—a single dose of Cytoxan caused 90% regression with a nadir 8 days later. B: Extensive bioassay data (intracerebral transplantation of counted control and treated cells) show that the single treatment quickly rendered 99.99–99.999% of the tumor cells incapable of indefinite proliferation. In a companion experiment (60 mg/kg Cytoxan), no viable cells could be detected 1 day after treatment, and complete tumor regression was achieved. Perturbation increased the rate of proliferation of viable cells. C and D: Thymidine indexes are a poor measure of tumor cell kill. Obviously, tumor cells having lost the capacity to proliferate indefinitely (*see* B) will incorporate tritiated thymidine for a while.

within a few years instead of a few decades—we must learn how to sample and assay some of these solid tumor cell populations for surviving fraction, before and after given doses or courses of therapy. If we do not, we will have to continue to work half-blindly because of what I have called the "Wilcox phenomenon" *(25, 47)* (*see* text-fig. 2) and because many of our clinical trials will require years to provide information that we need today!



Text-figure 3.—Effects of chemotherapeutic perturbation (Cytoxan) on the response of plasmacytoma-1 to arabinosylcytosine (Ara-C) (data of Dr. D. P. Griswold). ● = control; △ = Ara-C, 10 mg/kg, every 3 hours (× 8) on days 12, 16, 20, and 24; × = Cytoxan, 30 mg/kg, on day 7 only; and ○ = Cytoxan, 30 mg/kg, on day 7 only → Ara-C, 10 mg/kg, every 3 hours (× 8), on days 12, 16, 20 and 24. *a*) The single dose of Cytoxan administered killed >99.99% of the tumor cells (*see* text-fig. 2). *b*) Four courses of Ara-C initiated on day 12 only slightly inhibited tumor growth. (Greater inhibition was observed if such Ara-C treatment was initiated when the tumor was very small.) *c*) When Cytoxan was administered on day 7 only (rendering >99.99% of the tumor cells nonviable) and followed by Ara-C, there was a significant delay in tumor recurrence over that observed when a single dose of Cytoxan was given. Such a delay resulted from tumor cell kill. *d*) This observation has been confirmed repeatedly and is interpreted to mean that perturbation (Cytoxan) has left tumor cell populations which, for a while, were more sensitive to Ara-C.

EPILOGUE

A. Several questions were asked following the above presentation that deserve more thoughtful answers than probably were given on the spur of the moment.

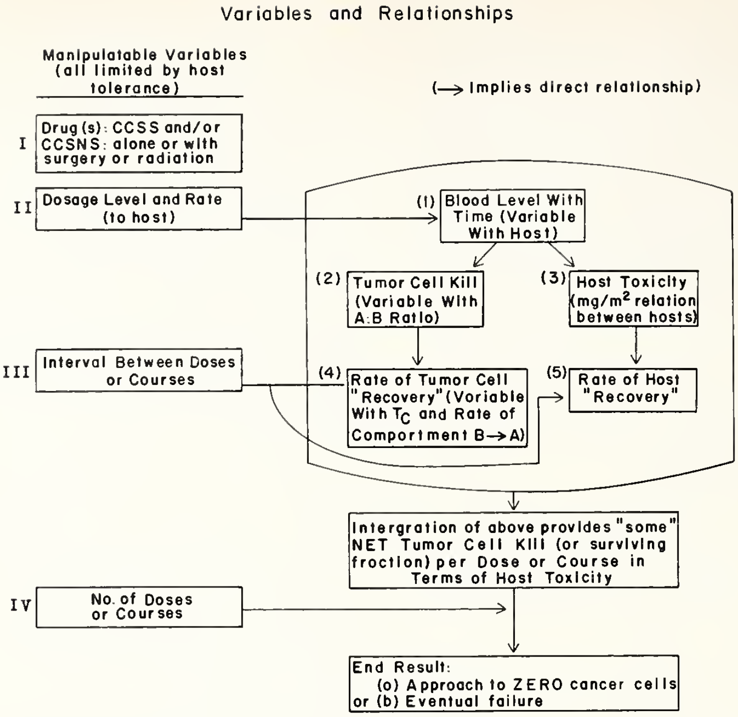
1) Why did you talk so much about "kinetics" in relation to chemotherapy rather than basic biochemistry, pharmacology, host toxicity, and rate of recovery, host immune response, etc.? The first reason is that I was asked to talk only about "Kinetic behavior versus response to chemotherapy" for an allotted time. A couple of quotations and text-figure 4 might suggest what I mean by "kinetics" and where this type of information fits and helps interdigitate important knowledge already provided by other disciplines. "It pleases me to imagine that the body of information being contributed by kineticists and cell biologists is providing a framework which links classical biochemical knowledge with that of cellular behavior, particularly the cell division function which is of paramount interest to the most practical cancer therapist" *(48)*. "To me one of the beauties of knowledge of the cell cycle is that it puts a temporal parameter, albeit only a median, on a series of biochemical events (DNA synthesis) which are peculiar to the cell replication function. Needless to say, the molecular events leading to functional DNA have long been a target of organic chemists engaged in synthesis of potential anticancer agents" *(48)*. I am most interested in applying "kinetic" information in the context of other knowledge and in a manner indicated in text-figure 4.

2) Is there any hope that information on the cell cycle characteristics of tumor cell populations can be of practical value in *a*) suggesting optimal regimens or schedules for specific classes of drugs or *b*) learning how to begin to translate optimal schedules from rapidly growing models to optimal schedules for less rapidly growing tumor cell populations with a wider ${}^{T}C$ range?

Data in table 6 might be interpreted as follows:

a) To "cure" some animals bearing relatively small numbers of L1210 leukemic cells (10^4–10^5) with an S-phase-specific drug, the interval between periods of "effective blood concentration" should be somewhat shorter than the median ${}^{T}S$ of the leukemic cell population.

b) Such treatment must be continued long enough for all cells with proliferative integrity to move out of


Variables and Relationships
Manipulatable Variables (all limited by host tolerance)
(⟶ Implies direct relationship)
I
Drug (s): CCSS and/or CCSNS: alone or with surgery or radiation
II
Dosage Level and Rate (to host)
(1) Blood Level With Time (Variable With Host)
(2) Tumor Cell Kill (Variable With A:B Ratio)
(3) Host Toxicity (mg/m² relation between hosts)
III
Interval Between Doses or Courses
(4) Rate of Tumor Cell "Recovery" (Variable With T_C and Rate of Compartment B⟶A)
(5) Rate of Host "Recovery"
Intergration of above provides "some" NET Tumor Cell Kill (or surviving fraction) per Dose or Course in Terms of Host Toxicity
IV
No. of Doses or Courses
End Result:
(a) Approach to ZERO cancer cells
or (b) Eventual failure

TEXT-FIGURE 4.—Variables and relationships pertinent to chemotherapy of any neoplasm in any host. CCSS = cell cycle stage specific; CCSNS = cell cycle stage nonspecific.

$G_1 + M + G_2$ and be exposed to effective concentrations of arabinosylcytosine (Ara-C) while they are in the vulnerable part of the S phase.

c) Although *median* ${}^{T}G_1 + {}^{T}M + {}^{T}G_2$ in early L1210 cell population is only about 4 hours, the extreme range of this period for viable cells within the population is ≥ 24 hours, depending on the degree of advancement of the disease.

d) The median ${}^{T}S$ of *advanced* human acute leukemia (and other advanced solid tumors) is about 1 day (table 4). Therefore, optimal schedules of Ara-C for acute lymphocytic or acute myelocytic leukemias might be those providing "effective blood concentrations" at intervals somewhat shorter than 20 hours. However, a major hurdle would appear to be the median $G_1 + M + G_2$ period of about 35 hours and a *much longer extreme range* in still viable leukemic cells. Providing effective blood concentrations at periods which would allow no leukemic cells to pass through S phase "unexposed," long enough to empty $G_1 + M + G_2$—without unacceptable toxicity to normal hematopoietic stem cells—does not appear too hopeful. This situation might be improved in acute lymphoblastic leukemia if optimal scheduling of Ara-C was initiated soon after remissions induced by, say, prednisone and vincristine, *i.e.*, when the leukemic cell number has been reduced by several decades and, hopefully, the extreme of ${}^{T}G_1 + {}^{T}M + {}^{T}G_2$ had been significantly reduced. Similarly, Ara-C would be expected to be somewhat more effective against other neoplasms after most tumor cells had been removed or killed *in situ* by other modalities or agents (text-figs. 2, 3).

The fact that optimal scheduling for methotrexate, 6-mercaptopurine, or 5-fluorouracil differs markedly from that of Ara-C or nucleotide reductase inhibitors is compatible with basic knowledge on the mechanisms of action of these agents. All of the therapeutic results, of which I am aware, indicate that the worst way to administer alkylating agents is in relatively low daily doses such as are tolerated for a month or months, *e.g.*, Burkitt's tumor.

B. Finally, I believe that the 1970's will be a period during which: 1) Agents with greater usefulness against some types of "tough," metastatic, solid tumors will come to hand, largely because we now know more about what the major problem is (was) and we have begun to learn how to design, select, and *use* such agents somewhat more effectively. 2) The long-term control of the most "drug-susceptible" human tumors (*e.g.*, choriocarcinoma, Burkitt's lymphoma, acute lymphocytic leukemia, Hodgkin's disease, or metastatic Wilms' tumor) will be improved in a stepwise fashion, because of better selection of agents and combinations and better scheduling (table 7). 3) Some additional types of disseminated human cancer will move to the more responsive categories because of better selection of drugs and combinations, better scheduling, and the use of chemotherapy before detection of gross recurrence after unsuccessful surgery or radiotherapy (table 7).

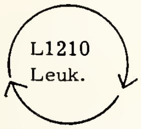
The above is far from a prediction of a sudden dramatic "breakthrough." Rather, it suggests that continued stepwise progress (possibly accelerated) seems feasible based on what already has been done by many basic scientists and research clinicians during the past decade.

Table 6

The Critical Importance of the Interval Between Periods of Effective Blood Concentration of Arabinosylcytosine and Response of Early L1210 Leukemia (a High Growth Fraction Neoplasm)

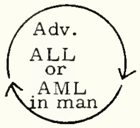
mg/kg/ dose	No. of doses	Interval Between Doses	Interval Between Doses During Which Blood Concn. Was <0.3 μg/ml (hrs)	% "Cures" in Animals Bearing Different Numbers of Leuk. Cells			Comment
				10^6	10^5	10^4	
15	8	10 mins	0	0	0	0	Periods of effective blood concentration were too short (i. e., 3-9 hours)
	8	30 mins	0	0	0	0	
	8	1 hr	0	0	0	0	
15	8	3 hrs	1	0	10	60	Optimal interval between 15 mg/kg doses = <9 hrs; optimal interval between periods of effective blood concentration = <7 hours
	8	4	2	-	10	70	
	8	5	3	-	20	60	
	8	6	4	0	10	40	
	8	7	5	-	30	40	
	8	8	6	-	20	30	
15	8	9	7	-	0	0	Interval between periods of effective blood concentration was too long!
	8	10	8	-	0	0	
	8	11	9	0	0	0	
	8	24	22	0	0	0	

S = 9 hrs (median)



$G_1 + M + G_2 = 3.8$ hrs (median)
with extreme range up to 24 hrs
or > depending on cell number

S = ca 20 hrs (median)



$G_1 + M + G_2 =$ ca 35 hrs (median)
with extreme range doubtlessly
much greater

Note: A similar type of schedule dependency is observed with other presumably S-phase specific agents (hydroxyurea, guanazole, and 5-hydroxy picolinaldehyde thiosemicarbazone, all nucleotide reductase inhibitors), but not with methotrexate, 6-mercaptopurine, or 5-fluorouracil, or with alkylating agents or agents which complex with DNA, regardless of cycle phase.

TABLE 7.—Status of clinical cancer chemotherapy [adapted from Luce, Bodey, and Frei (*37*)]

Best responses (remission rates) achieved by chemotherapy prior to 1967:
a) Advanced diseases, often after failure of other modalities
b) Not necessarily with optimal drug selection or optimal scheduling
c) "Objective" = 50% or greater regression in tumor volume, which implies far greater than 50% tumor cell kill (*see* text-fig. 2)

Disease entity		Comment (1970) (*44*)
	A. MAJOR BENEFIT	
1. Choriocarcinoma	75% complete; probable cure in most responders	90% complete; 80% survival at 5 years after diagnosis
2. Burkitt's lymphoma	60% objective; 15% long-term complete	Stage I and II: 90% complete; 90% survival at 2 years Stage III: 80% complete; 65% survival at 2 years
3. Acute lymphocytic leukemia in children	90% complete hematological; prolongation of median survival from 3 months (untreated) to 32 months; combinations improve effect	95% complete; 50% survival at 3 years
4. Hodgkin's disease	70% objective with single agents; 90% objective, 50–90% complete with combinations	Stages III and IV: 80% complete remissions; 50% survival at 4+ years
5. Lymphosarcoma and reticulum cell sarcoma	50% objective with single agents; 80% objective with combinations	
6. Multiple myeloma	35% objective	
7. Wilms' tumor	30–40% objective; also adjuvant to surgery	50% complete; 50% survival at 2 years
8. Neuroblastoma and retinoblastoma in children	60% objective; some long-term in infants	
9. Rhabdomyosarcoma	80% objective	
10. Chronic myelocytic leukemia	90% objective; probably no increased survival	
11. Chronic lymphocytic leukemia	50% objective	
12. Breast:		
(post castration or post menopause)	20% objective	
(late post menopause)	30–40% objective	
13. Ovary	30–40% objective	
14. Uterine body	40% objective	
15. Prostate (estrogens)	70% objective	
16. Testis	30–40% objective; 5% long-term (possible cure)	
	B. MODERATE BENEFIT	
17. Acute leukemia in adults	10–30% complete	
18. Large bowel tumors	15% objective	
19. Tumors of adrenal cortex	25% objective	
20. Head and neck cancers	20–30% objective	
21. Lung neoplasms	20% objective	
	C. MINIMAL BENEFIT	
22. Stomach, liver, pancreatic tumors	10% objective	
23. Melanoma	5% objective	Probably higher objective response in 1970
24. Differentiated sarcomas	5% objective	
25. Tumors of uterine cervix	5% objective	

Note: (1) What is it about disease entities 1, 2, 3, 4, and 7 which puts them in the "most drug-susceptible" category? Are they the "most rapidly growing" with the least range in ^{T}C in the still viable tumor cells? Diseases 1, 2, and 3 appear to be the most responsive to agents which kill cells in S phase.

(2) Has development of better schedules improved the response rate and the survival rate of patients with choriocarcinoma, Burkitt's lymphoma, and acute lymphocytic leukemia? Is the available methodology *for estimating* tumor cell kill following a given dose or course of treatment better for disease entities 1, 2, and 3 than many of the other neoplastic diseases listed? The answer to both of these questions appears to be "yes."

(3) Is the difference between an observed "complete" and "objective" remission the reflection of a quantitative difference in tumor cell kill? Probably "yes" in most instances, but again tumor mass behavior can be misleading because of differences in rate of resorption of dead cells and rate of proliferation of surviving cells—both of which affect rate and degree of regression and rate of recurrence.

(4) Is there any rationale or hope for improvement in the long-term survival rates in disease entities 1, 2, 3, 4, and 7, and the remission rates (and degree of remission) in those disease entities for which only 5–90% "objective" remission has been achieved in the past? The hope stems from improved performance (1, 2, 3, 4, and 7) over the past few years; the rationale rests on basic and clinical knowledge with respect to optimal selection of agents and optimal scheduling and the influence of "perturbation" on the sensitivity of the surviving tumor cells. Stepwise efforts toward such goals, if they are to be efficient, will require even greater cross-disciplinary understanding and collaboration than we have been able to achieve in the past.

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Limited Role of Cell Kinetics in Clinical Cancer Chemotherapy[1, 2]

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THE PAPER which Dr. Valeriote presented at this Symposium was a review of the work he and Dr. Bruce had done, and it appeared in the *Journal of the National Cancer Institute,* Vol. 42, No. 6, p. 1015, 1969. In it Dr. Valeriote made a case for the use of the model that they had used in which the effectiveness of a drug was related to its ability to reduce the cells which produced colonies, in the spleens of irradiated mice, following injection into such recipients. Using agents which are active against mouse tumors, and mouse tumors which are sensitive to several agents, they showed that improvements in the survival of tumor-bearing mice could be made using data on tumor and marrow cell kinetics obtained from their system. Since much attention and substantial funds are being used to pursue these concepts, it seems appropriate as part of the material of this meeting to apply a little critical judgment to the claims that have been made for the contributions that a better understanding of tumor cell kinetics can make to human cancer chemotherapy. It is my contention that such claims are largely untenable, being based upon broad generalizations from restricted data pertaining to models which are generally irrelevant to the human situation.

Basic Conceptual Errors

Most of the claims for the potential usefulness of cell cycle kinetics are based upon four erroneous assumptions. The first is that the rapidly growing mouse leukemia is a good model for the human situation. Thus, the concept that cytosine arabinoside would be best given by prolonged intravenous infusion in man arose because of the finding that this type of therapy was best for tumor-bearing mice. However, on clinical trial, no benefit can be shown for prolonged infusions in leukemic man of cytosine arabinoside over the drug given by once or twice weekly large pulse-doses. In addition, the concept that exposing the cells to such a "cycle-active" drug constantly for several generation times should result in a high cell-kill has not been shown at all for solid tumors in man, in which the drug is virtually ineffective, and cannot be made more effective by any modification of routes or duration of administration so far undertaken. Most human tumors, including leukemias, are vastly different biologically from transplanted mouse leukemias.

The second fallacy in the cytokinetic argument is that they neglect to recognize that the mouse data are obtained using drugs which are very active in the diseases studied. This is not the case in human cancers: Save for acute lymphocytic leukemias of childhood and a very few solid tumors, human cancers are highly resistant to the very same drugs that can cure many mouse tumors. The concept that you can improve the effectiveness of a good mouse drug by adjusting the dosage schedule to the kinetics of a sensitive mouse tumor would only find a counterpart in man if there were a number of reasonably effective drugs and reasonably sensitive tumors.

The third fallacy is that cytokinetic information can lead one safely to ignore biochemical differences between tumor cells, and if one patient with acute myelogenous leukemia fails to respond to a drug regimen that is effective in another, this difference will be found to be based more upon cell kinetic differences than biochemical differences such as differential drug metabolism. This point of view ignores the available evidence that, although the cell cycle kinetics when studied have been virtually identical for drug-sensitive and drug-resistant cells of morphologically identical type, these cells can usually be clearly separated biochemically, since the drug-resistant cell almost invariably has lost the mechanisms for drug uptake and retention which are present in the sensitive cell.

A final fallacy in the point of view of those who promote cell-cycle kinetics as paramount in chemo-

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[1] Substituted for a paper presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970, by Dr. W. R. Bruce, Dr. F. A. Valeriote, and Dr. H. Lin, Ontario Cancer Institute, Toronto, Ontario, Canada.

[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

therapy is their studied inattention to the cancer cell with drug resistance. Even the most sensitive of human leukemias will relapse in time in about 75% of the cases. Acquired drug resistance is therefore the major problem preventing the cure of lymphatic leukemia in children. In the human solid tumors, innate drug resistance is found in 75% of all patients with respect to all drugs, and at all times in the treatment course. Acquired drug resistance eventually overcomes virtually all responding solid tumors in time. All studies reported to date on the cell kinetics of solid tumors with different degrees of drug sensitivity have shown no basis in cell kinetic differences to identify a responding tumor type such as breast from a resistant tumor type such as lung. Similarly, after resistance appears, there are no regularly accompanying or characteristic changes in cell kinetics.

Cell Kinetics Does Not Explain Differences in Drug Sensitivity Between Normal and Malignant Cells

In the gastrointestinal tract, the rate of cell division, the cell cycle time, S-phase duration, and growth fraction of the normal mucosa overlap entirely the same values for colon cancer. Yet 25% of colon cancers can be effectively treated by 5-fluorouracil (5-FU), a finding which belies the importance of cell kinetics to chemotherapy. In addition, the same percentage of patients are found to respond to 5-FU regardless of whether the drug is given daily, weekly, or by courses, and independent of schedule, which suggests that inherent biochemical drug-metabolizing differences are more important in determining response than kinetics.

The same conclusion is true for tumors of the skin as compared with normal skin and the rate of division of normal bone marrow stem cells as compared with leukemic blast cells. In none of these instances can cell kinetics differentiate between malignant cells and normal cells, although differential drug sensitivity is the basis for effective chemotherapy in each instance.

Kinetics Cannot Tell Which Tumor Will Respond to Cycle-Active or Noncycle-Active Drugs

Cell-cycle kinetic data from mouse leukemias suggest that more rapidly dividing cells are more sensitive to cycle-active agents such as antimetabolites, while slower growing tumors respond better to noncycle-active agents such as the mustards and cytotoxic natural products.

However this concept fails utterly when applied to human cancers. For example, acute lymphoblastic leukemia is highly sensitive to a host of agents, while acute myelogenous leukemia (AML) is resistant to almost all of the same agents. There should therefore be early discernible and characteristic differences in their cell-cycle kinetics. In fact, there are none whatever, and no amount of schedule manipulation of agents such as 6-mercaptopurine (6-MP) has made them effective in AML. Such differences as do exist, however, can be easily correlated with innate biochemical differences between the cells; *i.e.*, 6-MP is phosphorylated to an active compound by many acute lymphatic leukemia cells and virtually no acute myeloblastic leukemia cells.

Similarly, rapidly growing human tumors such as Burkitt's lymphoma, choriocarcinoma, and melanoma, according to cell kinetic concepts, should resemble each other in having greater S-phase drug sensitivity than slower growing tumors such as chronic lymphocytic leukemia, breast cancer, and kidney cancer. Furthermore, the first group of fast-growing tumors should respond primarily to antimetabolites. In point of fact, we actually find:

> 1) Burkitt's lymphoma responds, not to antimetabolites, but best to alkylating agents, as well as do the slower growing lymphomas.
>
> 2) Choriocarcinoma responds, but to both cycle-sensitive agents, *e.g.*, methotrexate, and to noncycle-sensitive agents, *e.g.*, actinomycin D and Velban.
>
> 3) Fast-growing melanoma does not respond to antimetabolites such as 5-FU, but slow-growing breast cancer does.

While no cell kinetic data exist that explain these findings, there are data suggesting that the clinical responses observed are based on quantitative biochemical differences in drug handling of such degrees as to make the tissues qualitatively different in their responses to drugs.

Conclusions

Cell kinetic concepts are of virtually no current importance theoretically or practically in the chemotherapy of human malignancies. They may have a restricted role in suggesting improved schedules for known effective agents in known sensitive human tumors. Since there are at present only a few drugs which are very effective in a few patients with human tumors of specific types, primary emphasis should be placed upon developing biochemical assays which will identify responding patients and permit improved individualized therapy by "tailoring" drugs to patients. Secondly, increased effort should be given to the synthesis of new drugs based upon the drug metabolic pathways present in sensitive tumors. Cell kinetics has been a relatively unproductive research area and shows little signs of changing this position.

Possible Role of Cell Kinetics in Prediction of the Response to Cancer Therapy[1, 2]

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MANY CHEMOTHERAPEUTIC agents act at only one phase of the cell cycle and are totally ineffective against nonproliferating cells. Thus, the response of a tissue to a single injection of a cycle-dependent drug is determined by the proportion of cells in the sensitive phase of the cycle when the drug is injected into the tissue. If multiple doses are given, the intervals between injections might be so spaced that tumor cells, in a resistant phase at the time of one injection, appear as a partially synchronized wave in the sensitive phase at the time of subsequent injections. If cells of the limiting normal tissues proliferate at rather different rates, it is at least theoretically possible to obtain a greater killing effect in the tumor. In practice, this approach requires that cell-cycle phase durations of tumors and normal tissues be accurately known and that phase durations be relatively invariable. The presence of nonproliferating cells poses an additional problem in chemotherapy: Nonproliferating cells are resistant to many chemotherapeutic agents, but some probably retain the capacity to proliferate if their environment changes. Agents not cycle dependent might be used to attack these cells; however, the effectiveness of these agents depends on the concentration that can be achieved in the neighborhood of proliferating cells and, hence, on the position of these cells relative to the vascular system.

Variations in radiosensitivity within the cell cycle are much less than variations in sensitivity to drugs. Thus the optimal timing of fractionation in radiotherapy depends more on the rates of recovery and repair between fractions, and on increases in the oxygen tension of previously hypoxic cells, than on cycle-dependent variations in radiosensitivity. However, the relative rates of recovery of tumors and of the limiting normal tissues depend on their rates of proliferation; in addition, the "reoxygenation" of previously hypoxic cells may parallel the recruitment of nonproliferating cells into cycle during treatment.

It is relatively easy to obtain comprehensive information on cell proliferation in transplanted animal tumors; studies of one particular tumor are described below and illustrate how the spatial distribution of nonproliferating cells might influence the response to therapy. Specific predictions can be based only on detailed studies of human tumors and, for both ethical and practical reasons, these studies are possible only in selected patients. Shirakawa *et al.* *(1)* obtained kinetic parameters for subcutaneous metastases in 5 patients with human melanoma. Some of these data are presented below; they suggest causes for the rather poor response of melanoma to chemotherapy and also approaches to improve this response.

TERMINOLOGY AND EXPERIMENTAL METHODS

Tumor cell populations are often heterogeneous: The cells may have widely differing intermitotic times, and some (the nonproliferating cells) may not divide again in the untreated tumor. We therefore tried to obtain a distribution of intermitotic times rather than a mean or median value that may not be representative for many of the cells. The relative size of the proliferating population will be specified by the growth fraction. The nonproliferating population may include both end cells and clonogenic cells that do not re-enter mitosis in the unperturbed tumor.

The labeled mitosis technique provides the most sensitive method for estimation of the durations of cell cycle phases. A single injection of tritiated thymidine is followed by serial biopsies, and the proportions of labeled mitoses are determined from autoradiographs and plotted against time. In an

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[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

ideal cell population with constant phase durations, a series of rectangular waves would be seen: These waves are generated each time the cohort of labeled cells passes through mitosis, and the time between corresponding points on successive waves is equal to the cell cycle time. When the cycle time varies, the cohort becomes desynchronized and the curves are damped (*see* text-figs. 1A, 3A); the degree of damping is a measure of the spread in the distribution of intermitotic times. Curves may be analyzed by a computer method *(2, 3)* which assumes that phase times are lognormally distributed and determines the optimal fit to the data that is consistent with this assumption. The probable distribution of cell cycle times may also be obtained (*see* text-figs. 1B, 3B). The method is particularly valuable when there is no clearly defined second wave of labeled mitoses.

When the distributions of phase times are determined in a labeled mitosis experiment, the proportion of cycling cells in DNA synthesis may be estimated *(4)*. This would be the labeling index if all cells were in cycle. With nonproliferating cells, the measured labeling index is lower, and the growth fraction is equal to the ratio of the measured and expected labeling indexes. A more sensitive method (*see* text-figs. 2, 4) is to determine the labeling index at intervals during continuous thymidine infusion (or after repeated injections of thymidine). Continuous labeling curves approach 100% labeling even with nonproliferating cells, since the latter are produced from proliferating (and hence labeling) precursors; however, the rate of increase in labeling index is quite sensitive to the value of the growth fraction.

Cell Proliferation in Mouse Mammary Tumor; Spatial Variation in Kinetic Parameters

Chemotherapeutic agents reach tumor cells by diffusion from the blood vascular system, and their concentrations are probably lower in regions remote from a vessel. Since the effectiveness of a drug depends on its concentration and on the proliferative state of the cells, a study of cell proliferation in relation to the vascular system is essential to understand the probable results of therapy. The results of such a study, with the use of a transplanted mammary tumor in the C3H mouse, are presented below.

The tumor originated spontaneously in a mammary agent-positive mouse, and cell kinetics were studied in fourth generation isotransplants. Tumors were anaplastic and contained widespread regions of necrosis. In some regions were cylindrical "cords" of carcinoma cells surrounded by necrotic tissue, each with a straight and rather dilated capillary along its axis. This type of morphological structure was observed in other experimental tumors *(5)* and in several human epithelial tumors *(6)*; it defines the position of a cell in relation to its nearest blood vessel and is therefore convenient for the study of variations in cell proliferation with distance from a capillary.

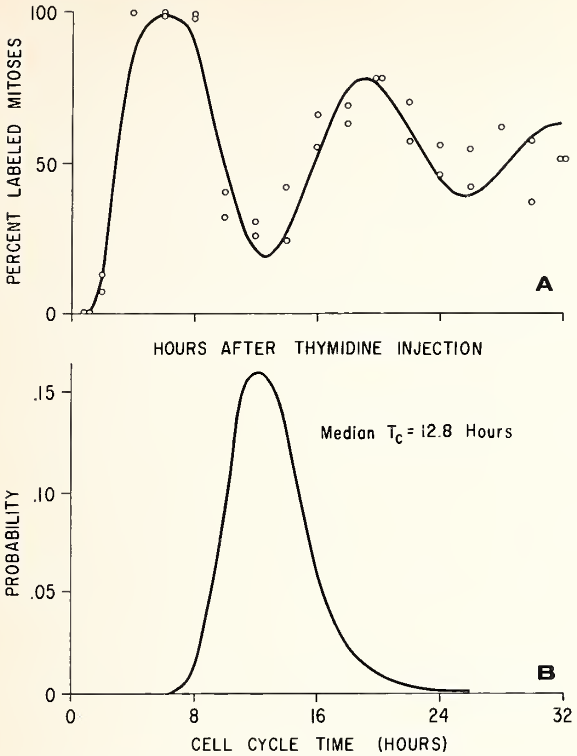
In tumor autoradiographs prepared from tissue samples taken 1 hour after injection of tritiated thymidine, the concentration of labeled cells clearly decreased with distance from the blood vessel. The mitotic and thymidine labeling indexes were therefore estimated in each of 3 regions of the tumor cords, defined by approximate trisection of the cord radius. Means and standard deviations are given below: The rate of cell proliferation was rather low in regions far from blood vessels.

	Region near blood vessel	Intermediate region	Regions near necrosis
Mitotic index	4.3 ± 0.4	2.5 ± 0.3	0.6 ± 0.2
Labeling index	50.0 ± 2.5	29.6 ± 4.8	10.3 ± 3.4

When the mean labeling index was measured at intervals after a single injection of tritiated thymidine, it increased with time; thus the progeny of well-labeled cells near a blood vessel move outward toward a region of necrosis.

The results of a labeled mitosis experiment are shown in text-figure 1A; a curve was computed to the experimental points. This curve had 2 clearly defined waves of labeled mitoses, and the corresponding distribution of intermitotic times (text-fig. 1B) was quite narrow; thus there is probably little variation in cell cycle time with distance from a vessel, which agrees with the results of an earlier study *(5)*. The median cell cycle time in this tumor was about 13 hours, and the median S-phase time was about 7 hours.

The results of a repeated thymidine labeling experiment are shown in text-figure 2. Animals were given injections of tritiated thymidine at 6-hour intervals, so that all cells entering the S phase should have become labeled. The variation in labeling index seen in text-figure 2 can be simulated by a theoretical curve only if the growth fraction is close to 50%. Two curves are shown, and their deriva-



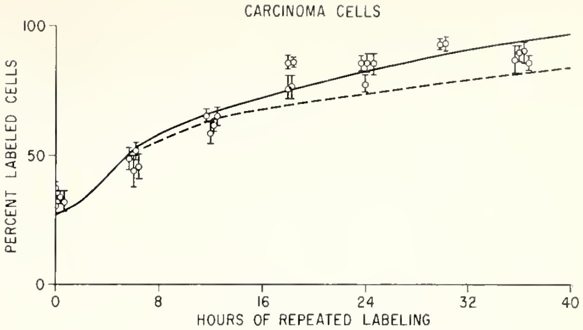
Text-figure 1.—A) Proportions of labeled mitoses in a mammary tumor of C3H mouse, at intervals after a single injection of tritiated thymidine. The curve was computed to fit the experimental points as described in text. B) Distribution of cell cycle times corresponding to computed labeled mitosis curve.

tion is based on different assumptions about the mode of cell loss *(4):* The solid line, which is the better fit to the data, was derived by the assumption that all cell loss was from the nonproliferating cells. This result and the observed variation in mitotic and labeling index suggest that, as cells moved away from a blood vessel, the probability of reentering cycle decreased and, ultimately, the cells became necrotic.

Cell Proliferation in Human Melanoma

In conjunction with Dr. Shirakawa, we studied cell proliferation in subcutaneous metastases of malignant melanoma *(1)* to find a rational basis for selecting and scheduling chemotherapy of melanoma.

Five patients received systemic injections of tritiated thymidine. Tissue specimens were obtained by aspiration or excision biopsies of the metastases.

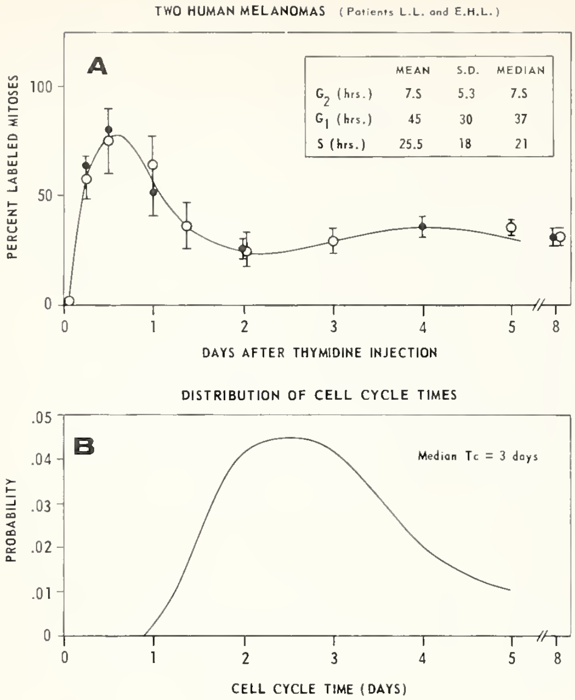


Text-figure 2.—Proportions of labeled cells in mammary tumor of C3H mouse after repeated injections of tritiated thymidine. Curves were derived by assuming a growth fraction of 50% with random cell loss (*broken line*), or with cell loss only from the nonproliferating population (*solid line*).

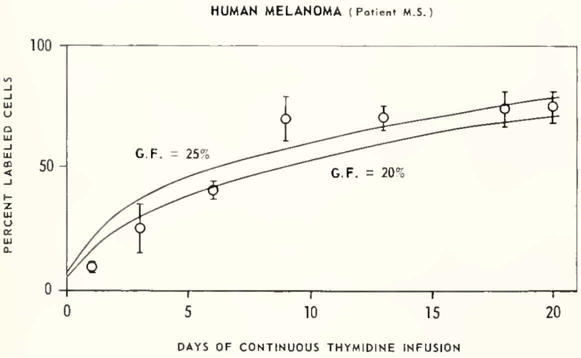
In each case, the consent of the patient was obtained. Spatial variations in labeling and mitotic index were studied in one patient. The labeling index varied from 3–12%, and parallel variations were seen in the mitotic index. However, among the 5 patients, there was little variation in *mean* labeling index (about 5%) and mean mitotic index (about 0.5%).

Serial biopsies were taken after a single injection of tritiated thymidine in 2 patients. The proportions of labeled mitoses were determined (text-fig. 3A). A single labeled mitosis curve was computed to the experimental points for both patients. The median G_2 and S periods were about 7.5 and 20 hours, respectively. There was no clearly defined second wave of labeled mitoses, and the distribution of intermitotic times was, therefore, rather broad (text-fig. 3B); the median of this distribution was about 3 days. An estimate of the growth fraction was obtained by a comparison of the mean measured labeling index of the 2 patients with that to be expected if all cells were in cycle. This estimate was about 30%.

Tritiated thymidine was infused continuously into a third patient for 20 days, and serial biopsies were taken at intervals during this infusion. The variation in labeling index is shown in text-figure 4. Curves were computed to fit these data by the assumption that the distributions of phase times were similar to those estimated in the labeled mitosis study for other patients. To obtain an acceptable fit to the points, it was necessary to assume a growth fraction of 20–25%.



Text-figure 3.—A) Proportions of labeled mitoses in subcutaneous metastases of 2 patients with melanoma. The curve was computed to the experimental points as described in text. B) Distribution of cell cycle times corresponding to computed labeled mitosis curve.



Text-figure 4.—Proportions of labeled cells in human melanoma during continuous infusion of tritiated thymidine. Curves were derived for growth fractions (G.F.) of 20 and 25%.

DISCUSSION

Skipper, Bruce, and others *(7, 8)* emphasized that the selection and dose scheduling of chemotherapy can be optimized by employing information with respect to the proliferative behavior of the tumor and, particularly, by exploiting differences in the cell kinetics of tumor tissue as compared to the cell kinetics of normal tissue *(9, 10)*. The median generation time for the melanoma cells was 72 hours, with a spread such that 90% of the cells had a generation time of less than 5 days. It has been demonstrated in experimental systems that optimal neoplastic cell destruction with cycle-active agents can be achieved by a course of treatment that continues through one or two generations of the neoplastic cells *(9)*. Subsequent courses are usually administered when the normal tissues (bone marrow and gastrointestinal stem cells) recover from the damage of the previous course. However, the effectiveness of these subsequent treatments in patients with melanoma would be much influenced by information concerning the rate at which the nonproliferating cells enter the proliferating pool. This information is now lacking. Under optimal circumstances with cycle-specific agents, the maximum cell destruction with a 10-day course would probably be no greater than 20%, since the growth fraction is only about 20%. A similar change in the mass of tumors would be marginally detectable with clinical techniques. Apparently indicated for the treatment of melanoma is the combination of a drug active in experimental systems against nonproliferating cells and a cycle-active agent.

The above considerations influenced our current approaches to the treatment of metastatic melanoma. The most recent program combines 3 agents: The first agent, vincristine, is cycle active; the second, 1,3-bis(2-chloroethyl)-1-nitrosourea, in experimental systems is effective against nonproliferating cells; and the third, imidazole carboxamide triazeno, produces progression delay in L1210 cells in the G_2 period but also, at least in tissue culture studies, may affect nonproliferating cells *(11, 12)*. The duration of and interval between treatments were designed as above. This program in preliminary analysis is producing a 40% objective response rate in patients with metastatic melanoma. The best recorded response rate for single-agent treatment is 20% *(13)*.

Because of the inaccessible location of many primary tumors and their metastasis (*e.g.*, lung cancer and gastrointestinal cancer), it will not be possible to obtain cytokinetic information of individual tumors before treatment. However, the study of selected patients with tumors in these categories (as in the above study of metastatic melanoma) may reveal information on the cytokinetics of that tumor category as a whole. Whereas variability within a category of tumor undoubtedly exists, marked variability for the melanoma patients did not obtain, and generalizations with respect to constructing chemotherapeutic programs seem reasonable. Cytokinetic information can be obtained in some tumors before treatment with regularity. Goodell, Young, and DeVita demonstrated that the double-labeling technique used on the bone marrow of patients with acute leukemia can supply rapid pretreatment information on the DNA synthesis period and on the generation time of the acute leukemic cells *(14)*. Hart *et al.* measured DNA sythesis of acute myelogenous leukemic cells of the marrow by an *in vitro* assay before and during treatment with various chemotherapeutic regimens *(13)*. The pretreatment DNA synthesis level in patients destined to respond to chemotherapy was 50% greater than that of nonresponders. There was considerable overlap; therefore, prediction for the individual case was not possible. The higher DNA synthesis rate suggests a shorter generation time and/or a higher proliferating pool consistent with a greater response to chemotherapy *(13)*.

The animal tumor studies described above also provide insight into some factors relevant to planning therapy. Thus, the physical relation between nonproliferating cells and the blood vessels from which drugs must diffuse has been illustrated with the mouse tumor described. While this result may not apply to all tumors, it is probably valid for human tumors having a morphologic structure similar to that of the mouse tumor described. The concentration of nonproliferating cells in regions remote from blood vessels renders these cells doubly resistant to therapy. Not only are these cells insensitive to cycle-dependent drugs, but they are in regions where only low concentrations of other agents can be achieved; being hypoxic, they are also resistant to radiotherapy. If nonproliferating cells were end cells, neither of these conditions would be relevant to therapy. However, the rather high doses of radiation required to cure the mouse tumor and the dependence of the cure dose on the oxygen status of the tumor *(15)* imply that some hypoxic, nonproliferating cells can retain their ability to proliferate.

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DISCUSSION

Chairman: I will ask the very broad question: What must we do to get the necessary cell kinetic data in man? What can we do for a carcinoma of the bowel or lung? I well remember when the late Dr. Murkle, who pioneered much of the theory for relating drug-dose schedule to cell cycle, first came as an experimental physicist. Dr. Murkle asked his physician why he was getting his drug at some fixed interval, and his physician could not give him a very good answer. Would the speakers in the audience be willing to comment on the notion that perhaps of equal importance to the kinetics of the cells in the tumor is the kinetics of the cells in the host, particularly of those organ sites of major toxicity?

Dr. Tannock: What you just said about the normal tissues is very important. I find it difficult to believe, with the diverse proliferation kinetics of the various normal tissues in the body, that we can ever devise a completely successful therapeutic regimen from a kinetic viewpoint alone.

Dr. Valeriote: The question you asked revolves around model building and what parameters we use in our model building. Essentially, the parameters used were the assumptions made, that a single cell is initially set; *i.e.*, a single cell, if it survives, will proliferate and kill the animal. We used our dose-survival curves to determine, after a given dose of drug, how many or what fraction of cells survives in mice given injections of a known number of malignant cells. One can thus estimate how many malignant cells are in the animal at death or at treatment. With this information, plus that on the fractional survival after a given drug dosage, one can determine how many cells should survive in that host after treatment. Knowing the doubling time of the tumor cell population, one can predict when this population will proliferate, reach a lethal number, and kill the animal. Then one can predict survival times and also how many cells should survive in the host after treatment. With statistics, one can predict what fraction of animals should survive if they bore so many cells or a certain fraction of cells survived in the population of mice.

Dr. Skipper: With regard to the problem of normal tissues, I think that, when our time is short like this, we tend to talk only about the neoplasms. But I should not like anyone to think that a great deal of work is not going on in the area of kinetics and overall toxicity. There is no use in killing all the cells in the tumor and not having a surviving host. We need the type of quantitation that Dr. Valeriote, Dr. Bruce, and associates in Toronto, our group, and several other groups have attempted to use for animals, *i.e.*, quantitation of what fraction of cells survives after a given dose of a given drug or a given course of a given drug. As a clinical research effort, we must try to develop methodology that will allow us to sample, in some tumors, the cells before therapy and that will enable us to assay somehow these cells as to viability. Although this sounds difficult, actually the system does not require proliferative integrity as the endpoint; all it has to do is correlate with proliferative integrity.

Dr. Heidelberger: There are three great eras in the religions of man. All of these religions had their high priests and their litanies. The first, which has been going on for several hundred years, I will not mention. The second major era of religion came up in the middle 1950's, the religion of molecular biology. The litany of molecular biology was: If we understood all about molecular biology, we would solve the cancer problem. Then, in the 1960's and 1970's, we had the religion of cell kinetics, whose chief high priests said that, if we understood all about the kinetics of human solid tumors, we would solve the cancer problem. Yet, lest we get carried away with our religious fervor on this subject, I would like to be an agnostic and point out the very obvious fact that there are other things to consider. Dr. Valeriote discussed various categories—hierarchies of compounds. I was pleased to see the compound I was acquainted with is in the top group. Dr. Valeriote attributed all the differences in the selective action to cell kinetics. This may be true, but there are also biochemical reasons for differences between actions of drugs on tumors of normal cells. So my first agnostic plea would be to biochemistry. Dr. Valeriote also showed a beautiful matrix in which, if you add 2 drugs together at optimal doses, you get optimal effects. I suggest that this sort of work was done 20 years ago, before we knew anything about kinetics, and approximately the same results were obtained. Those of you who get up early tomorrow, I will try to convince that not only drug delivery but drug metabolism is an extremely important component in this therapy problem. So while kinetics is tremendously important and great gains have come from it, other things should also be considered. Finally, Dr. Skipper's data clearly show, as I understand from conclusions of a meeting held here last week, that it is quite impossible for any of the drugs that we now know to have any effects on solid tumors. In breast cancers, the doubling time is 43 days; yet some of the drugs we have do produce 50% regression, which means more than 50% cell kill in breast tumors. So there are other factors beside kinetics which I would put in a plea for.

Dr. Skipper: Kinetic information has been very helpful in relating classic basic biochemical research of the type Dr. Heidelberger and many others have done over the years and to the knowledge of cell biology, pharmacology, and experimental therapeutic trial results. It has led to a better understanding of what has happened. If you go back, you can find Dr. Cohen's observations on unbalanced growth in the lowly *Escherichia coli* in much of what we see now. Compared to those biological and biochemical observations, I think it is fitting much better. We work a great deal now with spontaneous neoplasms. Spontaneous leukemia, spontaneous breast tumors, and 7,12-dimethylbenz[*a*]anthracene-induced breast tumors are not too far removed from the human.

Dr. Regelson: Oxygen tension should be considered in relation to the system involved. One should consider the developing embryo in regard to the neurological system.

Division occurs against the lumen in regard to the neural tube, unrelated to blood supply. Thus one thinks of nutritional factors related to oxygen.

Dr. Tannock: I do not think it is simply lack of oxygen that slows division. One of the processes going on is that these slowed cells are near regions of necrosis having very high concentrations of cytotoxic agents. For example, you can take fluid from the regions of necrosis and inject it into an animal and the fluid will kill the animal. Oxygen may be one factor involved in cell killing.

Dr. Heidelberger mentioned that we cannot cure any of our tumors or that therapy cannot be effective because many tumors have a doubling time of 44 days. I would like to point out that the doubling times of tumors are often very much longer than the rate of cell production within the tumors. Human tumors do not represent unrestrained growth, and we can measure the rate of cell death, or rate of cell loss to be precise, as a fraction of the rate of cell production. One surprising thing coming out of the study of human tumors is that, even if the tumor grows slowly, the turnover of the cells may be quite fast and the difference between the potential doubling time and the actual doubling time indicates a considerable amount of cell death.

Dr. Zelen: Suppose we have one cell which goes into the G_1 phase, then into the S phase, and finally into the G_2 phase and into mitosis—the end of this particular cell cycle. Just to make things simple, the cell goes back into cycle. In a whole population of cells, there are various durations of cell cycle and of DNA synthesis. Now, we are going to label the cells with a short pulse of isotope. The question comes up: Which cells are labeled? Not labeled randomly, the cells are labeled proportionately to the ratio of the DNA synthesis time to the duration of cell cycle time. So the population of cells being labeled does not represent the population of the tumor cells but is a biased sample of the tumor cells—so biased that the larger the duration of S the larger the ratio of S to T_C the more likely that the cell is going to be labeled. How important is this? If you have no cells that have random variation, *i.e.*, the duration of S phase and duration of T_C being exactly the same, it does not matter. On the other hand, if you have cells that have great variability, which is what we expect of human cells, this is going to be a very important phenomenon. And although one can get out all sorts of numbers, such as the duration of cell cycle times, S phase, etc., serious questions must be discussed about what they do represent because the labeled cells are essentially not typical of the cell population. You are labeling those cells that have relatively high values of S to T_C; you are missing completely those cells that have small ratios of DNA synthesis time to the duration of cell cycle.

Dr. Tannock: If I understand what you are saying, when you are labeling, you tend to label the fastest proliferating cell. We have good evidence that variation in the S phase is not very great; we can also do that from double-labeling studies and so on. There is a lot of variation in cell cycle, and the cells we tend to label are the fastest proliferating cells. But is that not what we are trying to say with labeling indexes? What we are saying is the labeling index is a measure of the rate of proliferation. Therefore, the unlabeled cells tend to proliferate more slowly or not to proliferate at all. I would answer you in terms of how we can analyze this population in the sort of experiment I was describing, in which you label the cells and follow them between mitoses. Clearly, the time between when you see a labeled cell in one mitosis and the next mitosis would depend just on T_C. Now if T_C were fairly short and constant, you would see two sharp waves; if it were very variable, you would see no second wave or you would see a very dampened one. Clearly, this dampening is a measure of exactly what you are talking about here, *i.e.*, the heterogeneity in the system.

Dr. Bertino: I was going to pursue this point of cell loss a little and also of some differentiation in tumor cells, because the assumption is that there is no differentiation. All you are seeing is cell mitosis at the end of your blood vessel; skin and gastrointestinal mucosa have the same kind of situation, with proliferation close to the blood supply. As the cells move out, they die. There is evidence that explains this: some differentiation. Cells are made which cannot re-enter the mitotic pool. Are you looking at this as a possibility as well?

Dr. Tannock: That is perfectly true. The tumors I have looked at are anaplastic transplants, but certainly I think that nutritional failure or a high concentration of the cytotoxic agent is not the only thing that leads cells out of cycle. Differentiation is another one, at least in primary tumors.

Dr. Friedel: As a first point, you have to distinguish between "perturbed" and "normal" tumors. In your kinetic studies of other organs, it seems obvious that parts of a tumor have different rates of DNA synthesis, at least in our experience with the liver. It seems likely also that you may have increased and decreased turnover times in these other organs which may be important when you are thinking about therapeutic effect.

A second point is: How are we going to get at the changes that occur in solid tumor growth as it tends to level off? If you were studying for the most part changes in so-called "log-phase" growth, you pick your parameters and transplant your tumors—which, although difficult, is relatively simple compared to the problem of how to study the slope as it changes when you go into depressed growth rates. How are we going to get into this area?

Dr. Tannock: You can get at it in animal tumors by doing just the sort of study that I described, at different times in the growth cycle. The answer seems to be that tumors grow successively more slowly because the amount of cell death increases; this is the main factor. There also appear to be slight changes in the growth fraction in tumors becoming necrotic. However, the cell cycle times stay relatively constant because the nutrient environment of the living cells probably does not change very much once the tumor reaches a certain size. You have a relationship established between blood vessels and necrosis, but what is probably happening is that the necrotic region is getting bigger, more cells are dying, and the rate of tumor growth is decreasing.

Dr. Heidelberger: Dr. Zelen raised a very important point which was really not suitably answered in my opinion. As Dr. Skipper pointed out, cells in culture tend to have approximately the same doubling times. We have 2 cells in our laboratory, the L1210 leukemia cell which has a doubling time of about 13 hours and the HeLa cell which, in proper medium, has a doubling time of about 16 hours; these doubling times are not identical but are very similar. Yet the S phase in the L1210 leukemia cell lasts 10 hours and that in the HeLa cell lasts only 4 hours. So here 2 cells, dividing at approximately the same rate, have quite different S phases.

Dr. LePage: I wanted to comment about the manner of drug distribution that Dr. Tannock went through with his micrographs. I think this will obviously depend on the type of drug being studied. Some drugs are activated, and others are concentrated in tissues. We studied one nucleoside with no activation nor degradation. This nucleoside is a water-soluble agent which lends itself to a study of distribution, and the kinetics of excretion of this nucleoside indicated that it was distributed uniformly in body water. So we were concerned whether this nucleoside would penetrate the necrotic areas of the tumor as well as the actively growing areas. We used an experimental system in which large subcutaneous tumors were allowed to reach a size where they had a necrotic center. The drug was then injected with a radiosulfur label, and studies were made, at intervals, on the concentration of the drug in the necrotic areas as opposed to that in the rapidly growing areas. There was a very small lag in the concentration in the necrotic areas, of perhaps 5 minutes, and equilibrium was established. That is, the concentration in the necrotic areas was exactly the same as that in the growing areas so that, while nutrition may vary as you go from areas of oxygenation, certainly some drugs would not obey this type of relationship.

Dr. Skipper: As one comment to Dr. Heidelberger's and Dr. Zelen's point, certainly thymidine indexes are useful values, but they can be misleading also for reasons other than you mentioned. When a cell is exposed to tritiated thymidine and is labeled, you have no evidence whatsoever that the cell from then on out is going to retain proliferative integrity. When it returns to G_1, it may sit there, eventually lose proliferative integrity, die, and then become resorbed. Even with all these variabilities, there is a correlation between response to chemotherapy and thymidine index, not only for S phase-specific agents but also for alkylating agents and other agents. Tumors with a thymidine index of 60 or 70% will be responsive to properly administered dosages. These are transplanted tumors that nobody likes. Tumors with a thymidine index of 2% or 5% are difficult to treat, as far as complete tumor cell eradication is concerned.

Dr. Valeriote: In relation to Dr. Heidelberger's comment, we are certainly aware that there is a biochemical basis for the action in these agents. Our approach is, as I indicated, initially cellular; we believe that cancer is a cellular disease and we are interested in finding a way in which we can destroy all cancerous cells. We have attempted to study drugs for which the biochemical modes of action are not known. I was not trying to make the point that, by adding 2 drugs you get an additive effect, which has been obvious for that length of time, but rather to state the fact that, by using these quantitative data, one can rationally come to a conclusion by which one can employ one or more of these agents in such a way as to eradicate a sizable population of the cells in the tumor-bearing animal. I am really not aware of people 20 years ago who have used chemotherapy to cure or obtain long-term survivors in animals bearing 10^8 or 10^9 malignant cells.

Chairman: If cell kinetics contributes nothing else than the correct timing, which the chemotherapists like to call the schedule of administration, then if we fail to use cell kinetics, we will continue to stay not in the 1970's or into the 1980's, but we will stay in the 1950's and 1960's when good chemotherapeutic practice and research consisted of administering for 42 days a new agent to patients with solid tumors and then looking for a 50% regression. We failed at that time because we did not realize the impact of what timing of drugs was doing or what it was not doing. If we fail to include cell kinetics in our planning—rational or even mathematical planning—for the administration of drugs, then we will continue to flounder. We will continue to develop dose schedules that are random, that are hit or miss, or that are based on what is called clinical experience or clinical judgment, and those of us who have been concerned with clinical research often recognize that one's experience, which he dredges up without having reviewed it carefully, is often 100% incorrect.

PANEL II

HORMONAL INFLUENCES

Some Factors Influencing the Uptake of Steroid Sex Hormones by Target Tissues[1, 2, 3]

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OF THE various factors influencing the uptake of steroid sex hormones by target tissue, the following may be most relevant to biological responsiveness: *a*) the concentration of unbound steroid in plasma and *b*) the concentration of receptor molecules in target tissue. As a basis for discussion, one may represent the target cell as a solution of receptor molecules contained in a dialysis bag which is immersed in plasma. When steroid hormone is introduced into the outer solution, the receptor molecules and the plasma proteins compete for the binding of the hormone. When equilibrium is reached, the concentration of unbound hormone on either side of the dialysis membrane will be the same. According to this model, the concentration of unbound hormone in the target cell is equal to that in plasma. The equilibrium binding of hormone within the target cell may be described by the Scatchard equation:

$$\frac{S_b}{S_u} \cdot \frac{1}{P} = k\left(n - \frac{S_b}{P}\right)$$

where S_b and S_u represent the molar concentration of bound and unbound steroid hormone, respectively; P, the molar concentration of the receptor molecule; k, the intrinsic association constant for each binding site; and n, the number of binding sites in the receptor molecule.

Neglected in this model are other factors that may profoundly influence hormone uptake, *e.g.*, the permeability of the target cell membrane (and intracellular membranes) to steroid hormone, the binding of hormone to macromolecules other than the receptor (particularly to enzymes catalyzing steroid transformation), and the partitioning of unbound hormone between the aqueous and lipid phases of the cell. Thus, the concentration of unbound hormone within the cell may not be identical with that in peripheral plasma: More likely it is some function of the latter.

The ratio of the concentration of receptor-bound hormone in target tissue to that of unbound hormone in plasma at steady state may provide an index of tissue uptake most relevant to biological responsiveness. The molar concentration of unbound steroid hormone in plasma can be readily calculated from measurements of steroid binding, as will be described. It may be possible to estimate similarly the molar concentration of receptor molecules in a given target tissue.

However, in our study *(1)* on tissue uptake of estrogen in women with breast cancer, tissue uptake was not thus defined. In this study, radioactive estrogen was intravenously infused at a constant rate over a prolonged period. Tissue uptake was defined as the ratio of the radioconcentration of ether-soluble estrogen radiometabolites in tissue to that of the ether-soluble material similarly in plasma at steady state. A high uptake of estrogen was reported in breast tumors, especially in a metastatic lymph node. This observation led us to study steroid-protein interaction as a mechanism partially responsible for steroid hormone uptake.

We selected human serum as a model medium for studying steroid-protein interaction preparatory to undertaking similar studies with tumor tissue. A component of human serum exhibiting a high binding affinity for testosterone was unexpectedly found during this investigation *(2–5)*. The so-called testosterone-binding component or protein binds other 17β-hydroxysteroids, notably 17β-estradiol *(5)*. The testosterone-binding protein is distinctly different from the corticosteroid-binding globulin as a chemical entity and in its steroid-binding properties *(3)*; the latter protein binds progesterone as well as corticoids *(6)*. The testos-

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[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

[3] Aided in part by Public Health Service research grant CA 11628–01 from the National Cancer Institute and by research grant GB-15842 from the National Science Foundation.

terone-binding protein and corticosteroid-binding globulin occur concomitantly *(3, 6)* in increased amounts in advanced pregnancy, apparently in response to an increased production of estrogen; the plasma levels are similarly elevated in men with prostatic cancer receiving estrogen.

The plasma concentration of unbound testosterone appears to be only a fraction of the total (bound plus unbound) hormone. The following calculations *(7)* indicate that the testosterone-binding protein lowers the plasma level of unbound testosterone considerably below that expected from albumin binding alone.

The plasma level of testosterone-binding protein is calculated from binding measurements with our Sephadex assay technique *(2)*. This technique is based on the principle of equilibrium dialysis but uses Sephadex G–25 in a batchwise fashion on a semimicro scale. One simply notes the volume of serum required for 50% steroid binding in a Sephadex assay tube containing 1 ng of radioactive testosterone; as little as 5 μl of human pregnancy serum may be required in this rapid assay. The following equation *(7)* gives the concentration of testosterone-binding protein in the phase ($v_e = 1.5$ ml) external to the Sephadex gel in the assay tube:

$$n_1P_1 = (S_b + 1/k_1)\,(1 - n_2k_2P_2)$$

where P_1 is the molar concentration of the testosterone-binding protein, and n_1 is the number of binding sites in the protein molecule. (The value for n_1 has not yet been determined; it is probably equal to 1.) The other symbols are defined as follows. S_b is the molar concentration of bound radioactive testosterone and is equal to 0.08×10^{-8} M, a constant characteristic of the assay system; and $1/k_1$ is the dissociation constant of the complex of testosterone with the testosterone-binding protein and is equal to 0.22×10^{-8} M at 25°C. The term $(1 - n_2k_2P_2)$ corrects for albumin binding: P_2 is the molar concentration of albumin in the assay system; and n_2k_2, the equilibrium constant of association for the testosterone-albumin complex, is equal to 4.2×10^4 M at 25°C. The above equation thus gives the n_1P_1 value for the assay system.

The n_1P_1 value for undiluted serum is obtained by multiplying the n_1P_1 value for the assay system by the dilution factor, v_e/v_s, where v_s is the volume of undiluted serum, *e.g.*, 0.005 ml, introduced into the assay tube, and $v_e = 1.5$ ml as mentioned above. The dilution factor in this instance is 1.5 ml/0.005 ml = 300.

The serum concentration of total (unbound plus bound) testosterone, S, may be readily determined by competitive protein-binding analysis by use of pregnancy plasma as a source of testosterone-binding protein, as described by August *et al. (8)*. A fair approximation of the serum concentration of unbound testosterone, S_u, is obtained from the following equations *(7)* applicable to undiluted serum:

$$\frac{S_b}{S_u} = \frac{k_1(n_1P_1 - S)}{n_2k_2P_2}$$

and

$$S_u = \left[\frac{1}{1 + \left(\frac{S_b}{S_u}\right)}\right] S$$

where S_b/S_u is the ratio of bound-to-unbound testosterone in undiluted serum; the other symbols have already been defined.

The n_1P_1 values for testosterone-binding protein are of the order of 8×10^{-7} M and 1×10^{-7} M in late pregnancy serum and normal male serum, respectively. The corresponding S values for total testosterone, based on the report of August *et al. (8)*, are of the order of 0.5×10^{-8} M and 2×10^{-8} M, respectively. On substituting these values (and those for k_1 and $n_2k_2P_2$) in the above equations, we find that only 0.27% of the total testosterone in late pregnancy serum is unbound, and 1.7% of that in male serum is unbound. If the testosterone-binding component were altogether lacking in male serum, 4.5% of the total testosterone would be unbound. The role of the testosterone-binding component in regulating the level of unbound testosterone, and presumably therefore the physiologically effective level of the hormone, may thus be appreciated. The markedly elevated levels of testosterone-binding protein evoked on estrogen administration, as in the treatment of men with prostatic cancer, may thus significantly reduce circulating androgenicity.

This brief account is intended to illustrate a conceptual approach to the problem of breast cancer. This approach relies on a biochemical methodology for the detection and ultimate isolation of receptor molecules from normal and cancerous breast tissue. It assumes that the receptor is a macromolecule exhibiting a high binding affinity for specific steroid sex hormones. According to this hypothesis, normal breast tissue should contain receptors for both estrogen and progesterone, since these hormones act synergistically to stimulate normal growth and development of the breast. According to Lyons *et al.* *(9)*, normal breast growth requires the concerted action of a triad of hormones: prolactin, estrogen, and progesterone. Thus, for a target tissue such as normal breast, the receptor theory has many interesting ramifications. How much more so when considering the course of development of breast cancer! Is the concentration of the individual receptors for hormones (steroidal and nonsteroidal) higher than that in noncancerous breast? Is the pattern of hormone receptors significantly different? Have new receptors, *i.e.*, with different binding properties, come into prominence? As basic information of this sort becomes available, the prediction of response to endocrine cancer chemotherapy may be made on a firmer theoretical foundation.

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Estradiol Uptake by Induced Rat Mammary Tumors and Its Implications for the Treatment of Breast Cancer[1, 2, 3]

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IT HAS long been known that ovarian hormones influence the growth of normal and neoplastic mammary glands. Since 1896, when Beatson successfully treated two cases of human breast cancer by oophorectomy, endocrine ablation has been a standard treatment for this disease, although it is only successful in about 50% of cases. It is obviously desirable to be able to select the cases which would respond to such treatment before it is done, and such selection depends on the establishment of the characteristics of hormone-dependent tissue.

In recent years, radioactive hormones of high specific activity have become available, which has made it possible to investigate occurrences very early in the response of a tissue to a physiological dose of a hormone, *i.e.*, the uptake of a hormone by a tissue and its localization and metabolic fate when it has been taken up. Jensen and Jacobson *(1, 2)* showed—and it has since been confirmed by many other workers—that the so-called target organs, such as the uterus and vagina, can retain injected estradiol more than nontarget tissue like muscle or metabolizing tissue like liver. There are two other characteristics of estradiol uptake in the target tissues: It remains as free estradiol rather than being metabolized to estrone or to conjugates, and it appears to be finally localized in the nucleus and bound there by a specific receptor which is protein in nature.

In the investigation of the mechanism of action of hormones in breast cancer, much use has been made of the mammary tumors induced in rats by 7,12-dimethylbenz[*a*]anthracene (DMBA) first described in 1961 by Huggins, Grand, and Brillantes *(3)*. These tumors have some of the characteristics of some human breast tumors; most regress after ovariectomy or hypophysectomy, and they respond to treatment with androgens.

Jungblut, DeSombre, and Jensen *(4)* and King, Cowan, and Inman *(5)* showed that these hormone-responsive tumors behave like target organs for estrogens, in that they take up and retain estradiol, the estradiol does not appear to be metabolized, and much of the estradiol remains in the nucleus. Incidentally, all this suggests that estrogen acts directly on the mammary tissue, and not only indirectly, as, for example, by the stimulation of secretion of prolactin from the pituitary.

As I have mentioned, a few of these DMBA-induced tumors are not hormone responsive and continue to grow after ovariectomy. Histologically, they are not distinguishable from the responsive tumors. It was therefore of interest to compare the behavior of these tumors as regards the uptake of injected estradiol with that of the responsive tumors [Mobbs *(6)*].

Tumors were induced in female Sprague-Dawley rats by the gastric instillation of DMBA. When the tumors were growing steadily, the tumor-bearing rats were given subcutaneous injections of approximately 0.1 μg/100 g body weight of tritiated estradiol of high specific activity. After an interval ranging from 20–140 minutes, the tumor(s) and thigh muscle were biopsied, and the animals were ovariectomized. Part of each tumor biopsy specimen was used for histological examination, and the remainder and the whole of the muscle biopsy specimen were used for the estimation of radioactivity. The response of each tumor to ovariectomy was evaluated from the growth curve of the part of the tumor remaining in the animal after biopsy and by histological examination of the tumor at autopsy approximately 3 weeks after ovariectomy. Radioactivity was estimated in "free steroid" and "water-soluble" extracts of replicate samples of

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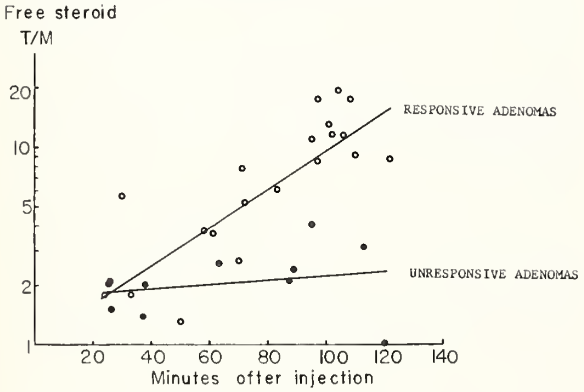
[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

[3] This work was done at St. Mary's Hospital Medical School, London, England, and at the Cancer Research Centre, University of British Columbia, where it was supported by the British Empire Cancer Campaign and the National Cancer Institute of Canada, respectively.

each tumor and muscle biopsy specimen. Since more than 95% of the radioactivity in the tumor samples was in the free steroid fraction, the results presented here are based on this fraction only.

As expected, muscle did not retain the radioactivity, and 100 minutes after injection the amount of radioactivity present was negligible. The pattern of uptake by tumors which did not regress after ovariectomy was similar to that of muscle, whereas the responsive tumors retained much more radioactivity. In text-figure 1, the results are expressed as the ratio of the concentration of the radioactivity present in the free steroid fraction of the tumor biopsy specimens to that present in muscle from the same animal (T : M). One hundred minutes after injection, the T : M ratio for the responsive tumors was in the range 8–20, whereas that for the unresponsive tumors did not rise above 4.1 for the whole period investigated. Determination of the T : M ratio could therefore provide a basis for the prediction of response to ovariectomy. An *in vitro* predictive test for this tumor has not yet been devised, although Terenius *(7)* studied the *in vitro* uptake of estrogens by hormone-responsive, DMBA-induced rat mammary tumors.[4]



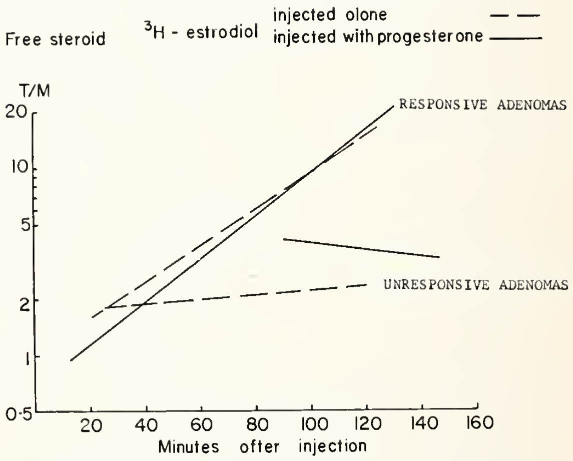
TEXT-FIGURE 1.—Ratio of radioactivity present as free steroid in biopsy specimens of mammary tumors (T) to that in biopsy specimens of muscle (M). (Although labeled adenomas because they do not metastasize, these tumors are identical with the Huggins carcinoma.)

Several studies of the uptake of estrogens by human breast cancer have suggested that human tumors behave in a similar way, but only one published study to date has related uptake to response. Folca, Glascock, and Irvine *(8)* found that a high T : M ratio of radioactivity 6 hours after injection of tritiated hexestrol was related to a positive response to subsequent adrenalectomy. Obviously, an *in vitro* test would be preferable, and this is under investigation in Professor Jensen's laboratory.

To return to the DMBA-induced rat tumor, there there is considerable evidence that progesterone, particularly when combined with estrogen, has a function in controlling induction and growth. Thus, an investigation was done similar to the one already described. But, in this investigation, ^{14}C-progesterone was injected simultaneously with tritiated estradiol, so that the uptake of progesterone and any effect progesterone might have on the uptake of estradiol could be investigated at the same time *(9)*.

Progesterone itself was not concentrated either by the responsive or by the unresponsive tumors and did not alter the pattern of uptake of estradiol by the responsive tumors. However, the T : M ratio of the unresponsive tumors tended to be higher when progesterone was administered with estradiol than when estradiol was administered alone (text-fig. 2).

These experiments indicate that progesterone, unlike estradiol, does not need to be retained by the tissues to exert its effect. But they also suggest (which, however, needs to be confirmed) that, in some way, progesterone facilitates the uptake or retention of estradiol by unresponsive tumors. Jungblut *et al.* *(4)* found that progesterone in-



TEXT-FIGURE 2.—T : M ratios for tissue biopsy specimens from animals given injections of ^3H-estradiol alone compared with those from animals given injections of ^3H-estradiol and ^{14}C-progesterone.

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[4] *See also* "Note," page 36.

creased slightly the uptake of estradiol by uterus and tumor of ovariectomized rats, and the extent of the increase depended on the dose of progesterone. Terenius (*10*), on the other hand, found that pregnancy or progesterone treatment *in vivo* or *in vitro* did not affect the uptake of estradiol by DMBA-induced tumors *in vitro*. These discrepancies may be due to differences in the doses of progesterone used. Such experiments may be relevant to the treatment of breast cancer with progestins.

As mentioned above, the DMBA-induced tumor responds somewhat to treatment with androgens. One possible mechanism for the inhibition of growth by androgens might be the prevention of uptake of estrogens by the tumor cells. As regards human tumors, the evidence on this point is conflicting. Deshpande *et al.* (*11*) reported that dromostanolone depressed the uptake of estradiol by human breast tumors *in vivo*, and this was also reported for tumor nuclei *in vitro* [King *(12)*], but this was not confirmed by another group of workers [Braunsberg *et al.* (*13*)]. The Huggins tumor was used to investigate this problem. Tumor-bearing rats were divided into 4 groups. All doses were per 100 g body weight. The rats were treated as follows:

Group 1: Rats were not pretreated with testosterone. They were given injections of 0.1 μg ^{3}H-estradiol and 0.1 mg testosterone at separate subcutaneous sites.

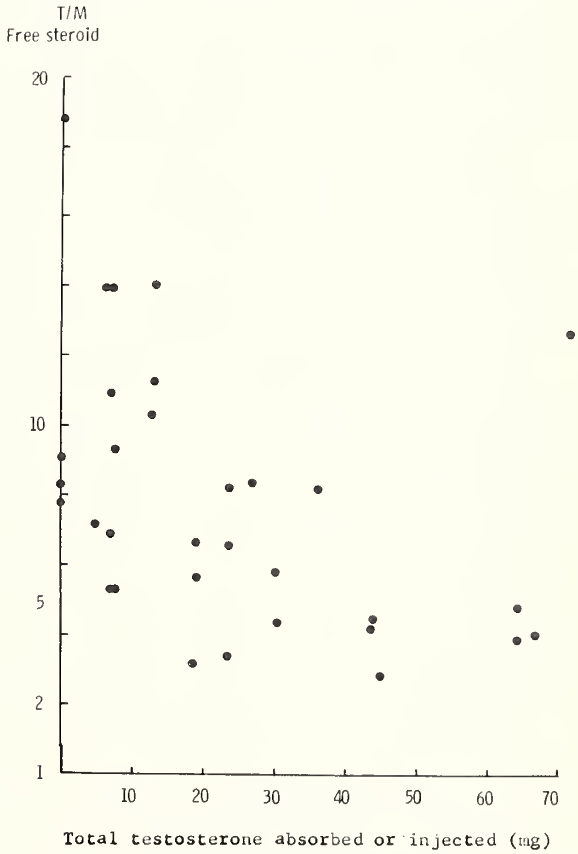
Groups 2 and 3: Rats were given injections of 0.2 mg and 1.0 mg testosterone, respectively, 5 times weekly, for 10–30 days before being given injections of ^{3}H-estradiol and testosterone as in group 1.

Group 4: Rats received subcutaneous implants in the dorsal neck of 2 pellets of testosterone, each weighing approximately 30 mg. After 14–39 days, they were given injections of ^{3}H-estradiol. After tissue biopsy specimens were taken, the testosterone pellets were removed, dried, and reweighed.

The response to treatment was evaluated by following tumor growth and by histology at autopsy. None of the treatments was wholly effective. The most successful groups were 3 and 4 which gave definite regression with 6/15 tumors. Response was not easily evaluated; regressions occurred after several days, unlike those in response to ovariectomy, which are rapid and definite, and some could have been spontaneous. The histological appearance of the tumors at autopsy did not always correlate well with the growth curve.

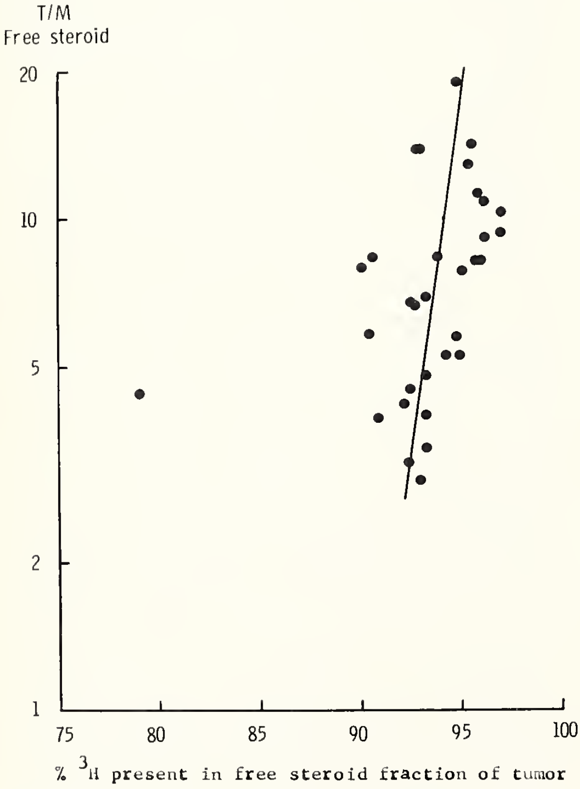
Biopsy specimens were taken 80–120 minutes after injection of ^{3}H-estradiol, for estimation of radioactivity and histological examination. The T : M ratios from group 1, in which the rats were given injections of testosterone at the time of ^{3}H-estradiol injection, were not different from those already described for rats given injections of estradiol alone. Therefore, it is unlikely that testosterone directly affects the uptake of ^{3}H-estradiol by tumor tissue. The mean and range of the T : M ratio in animals which had been pretreated with testosterone were somewhat lower than those in animals not pretreated, but the differences were not significant. However, the ratio in animals which had received a total dose of more than 15 mg of testosterone tended to be lower than that in animals which had received less than 15 mg (text-fig. 3).

It has been mentioned that, when estradiol is injected alone, most of the extracted radioactivity is in the free steroid fraction—in the case of the tumor, more than 95%. But it was found that, in



TEXT-FIGURE 3.—T : M ratios from tissue biopsy specimens of animals treated with testosterone before or at the time of ^{3}H-estradiol injection.

some tumors from animals treated with testosterone, the water-soluble radioactivity was unusually high (text-fig. 4). The slope of this regression line was just significant ($0.05 > P > 0.02$) and suggested that the lowering of the T : M ratio may have been connected with an alteration of metabolism of estradiol caused by the testosterone treatment. This needs confirmation and further investigation to see if such an effect on estradiol metabolism occurs in the tumor itself or, for instance, in the liver. Jellinck and Garland *(14)* showed that testosterone can affect the metabolism of estradiol by rat liver microsomes *in vitro,* but, as far as I know, this has not been confirmed *in vivo.* It might be interesting to try to correlate the antitumor activity of various androgens with the ability of androgens to affect estradiol metabolism. King *et al. (15, 16)* also found that these tumors have a varying ability to metabolize testosterone itself, and this may alter the effectiveness of testosterone as a treatment and may account for the great variation in response of these tumors to testosterone treatment.



TEXT-FIGURE 4.—T : M ratios and percentages of radioactivity in the "free steroid" fraction of tumors from animals represented in text-figure 3.

Although it is unwise to extrapolate too freely from the results of animal experiments to the possible mechanism of action of hormones in human breast cancer, it is believed that such experiments may provide useful guidelines for the investigation of hormonal action and also for the establishment of tests for treatment and prognosis.

Note: Since this Symposium was held, a paper by Sander and Atrramadal *(17)* has come to hand. This *in vivo* study confirms the observation that hormone-responsive, DMBA-induced mammary tumors of the rat take up and/or retain estradiol more than unresponsive tumors. Autoradiography demonstrated some labeling with ^{3}H-17β-estradiol in both types of tumor. Sander *(18)* also studied the uptake of estradiol *in vitro* by hormone-responsive and unresponsive, DMBA-induced rat mammary tumors with and without an estrogen antagonist. The latter inhibited estrogen uptake by responsive tumors, but the unresponsive tumors were not affected. A further report by Sander *(19)* described the incubation of human breast tumor and muscle biopsy specimens in medium containing ^{3}H-estradiol, followed by estimation of the amount of radioactivity present per mg protein. Of the 25 pairs of tissue investigated, 9 showed a T : M ratio > 3; the remainder had a ratio < 2.5. A similar study was carried out by Johansson, Terenius, and Thorén *(20)*.

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Some Basic Difficulties in Attempting to Predict Response to Therapy by Endocrine Assays[1, 2]

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SOME 10 years ago we described a method for differentiating between patients who responded to adrenalectomy or hypophysectomy and those who did not. The responders excreted large amounts of urinary 11-deoxy-17-oxosteroids relative to the amounts of 17-hydroxycorticosteroids (17-OHCS). Nonresponders excreted relatively low amounts of the androgen metabolites. The steroid assay results were combined in a discriminant function as follows: 80−80(17-OHCS, mg/24 hours) + aetiocholanolone (μg/24 hours). In a retrospective study based on some 40 patients, it was found that patients with negative discriminants tended not to respond to treatment, whereas those with positive discriminants generally responded well *(1)*.

At that time we hoped that the steroid assays would be of practical use in selecting patients for treatment, so that those patients who seemed to have little chance of a good response could be spared unnecessary major surgery. We are now sadder and wiser. It took 2 prospective clinical trials and another 8 years' work before we started to use the steroid assays in the day-to-day management of patients with metastatic breast cancer *(2)*.

A discussion of some of the difficulties we have encountered during this period might be useful at this Conference. Some of these difficulties are general ones and might be expected in any work on prediction of the results of therapy using endocrine assays. Others have only become apparent as our own work proceeded.

Lack of Basic Endocrinology on Growth and Maintenance of Normal or Neoplastic Human Breast Tissue

The lack of basic information has meant that most workers have investigated a particular endocrine aspect of the problem, using either information gained from studies on animal models or an intuitive approach. Since work with laboratory animals strongly implicated the estrogens either as carcinogens or as compounds on which the tumors depended, much effort has been expended on measuring the urinary metabolites of estradiol-17β in patients with breast cancer. The results are inconclusive, and there does not appear to be a single center where the type of treatment given to a patient is determined or even influenced by the results of measurements of estrogen status. Indeed, a tentative case can be made that, in at least some patients, the estrogens may be of minor importance as far as hormone responsiveness is concerned and that pituitary hormones are more directly involved *(3)*.

There is still no clue concerning the relationship of the discriminant to response to treatment. The urinary androgens, on which the main weight of the prediction rests, are derived in large part from plasma dehydroepiandrosterone, androsterone, and their sulfates. The physiological function (if any) of these compounds in normal or neoplastic breast tissue is obscure. These compounds are not even active androgens. The most likely explanation is that the amounts of these compounds found in the urine are an indirect marker for the production of other and more active hormones. For example, Adlercreutz *et al. (4)* found a direct correlation between urinary androgen and estrogen excretion in normal premenopausal women, which implies that androgen and estrogen excretion is linked. Wang and co-workers (unpublished data) found that sebum production is correlated with the plasma levels of dehydroepiandrosterone sulfate (DS). Again, the implication is that the amounts of DS secreted are in phase with the secretion rates of other primary hormones.

Variation of Remission Rate With Treatment and Unknown Mechanism of Treatment's Action

Some workers assume that all forms of endocrine therapy are equivalent. For example,

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[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

patients responding to any one of a variety of treatments—such as oophorectomy, adrenalectomy, hypophysectomy, or additive therapy with androgens or estrogens—may all be classed as having "hormone-responsive tumors." In the broadest sense this is true, but failure to define the hormone can lead to much confusion. There is evidence that patients who do not respond to one treatment (such as androgen or estrogen treatment) may still subsequently respond to ablative therapy. A small proportion of patients who have been treated by oophorectomy and adrenalectomy and who have progressive disease may still respond to hypophysectomy. These patients would be classed as unresponsive on the one hand and responsive on the other.

Therefore, a method for the prediction of response to one treatment may be virtually useless for the prediction of the results of another treatment.

If we assume that some 40% of patients have tumors that will respond to the most efficient treatment (hypophysectomy) and that these patients can be identified by a predictive test such as the discriminant function, then the more inefficient the treatment, the greater the degree of misclassification.

Even with a given treatment, results obtained by different surgeons may vary considerably, especially if different operative techniques are used. This factor is difficult to document, but the literature describing the earliest attempts at pituitary destrucion with radioactive substances brings this point home.

Heterogenicity of Patients With Breast Cancer

Biochemists tend to see the life of human beings in murine terms. Some of them appear to imagine that patients with breast cancer are as standardized as inbred strains of mice. There must be important biological differences between patients whose survival from diagnosis is measured in months and those who have a recurrence of tumor 30 years after mastectomy. Yet all too often the only information given is that the patients studied had "breast cancer." When patients are standardized as far as possible for some of the more obvious variables, the predictive efficiency of the discriminant function varies considerably for different groups. For example, the discriminant appears to be effective for distinguishing between women treated by hypophysectomy, who have had a free interval of less than 2 years, but it is useless when women with long, free intervals are considered *(2)*.

Most papers dealing with the relation between various endocrine parameters and response to treatment contain little or no information on the disease status of the patient, beyond the fact that the patient is premenopausal or postmenopausal. This is partly the fault of the authors who do not seem to appreciate that they are dealing with a heterogeneous population and partly the fault of editors who do not like to see paper "wasted" by printing tedious clinical details. In fact, the conventions of scientific publication are a distinct handicap in the dissemination of essential information. The great variability between patients and in the clinical course of their disease leads to another problem. This is that few centers see a sufficient number of patients to select a reasonably homogeneous group for study.

If the type of patient to be studied is narrowly defined, the investigation may take several years, and during this time it may be extremely difficult to keep some of the clinical variables constant. For example, if a new chemotherapeutic agent became available during the course of the trial, it would be unethical to withhold the new agent from patients with progressive disease, and this means that survival times may not then be comparable throughout the whole group of patients. If large numbers of patients are studied over a relatively short period, almost inevitably they will be extremely heterogeneous. One of the drawbacks in taking the generality of patients is shown by the work of Armitage and his colleagues *(5)* who examined the predictive value of a number of variables, such as the discriminant, free period, menopausal status, tumor size, etc., in some 200 patients with advanced breast cancer treated by endocrine ablation. There would be very few patients in most of the groups standardized for disease status. In some of these groups the discriminant, in combination with clinical factors, appears to afford a highly efficient method of prediction of the results of therapy. In others, none of the variables provided any worthwhile information. However, the small numbers in the individual groups precluded any degree of certainty that prediction of response would be effective on a prospective basis.

The Endocrine Pathways to Carcinogenesis May Be Multidirectional

A prospective trial on the relation between the excretion of steroid metabolites in the urine and the subsequent diagnosis of breast cancer was set up in 1961 *(6)*. The preliminary findings are consistent with the idea that there may be several abnormal endocrine patterns leading to, or associated with, the appearance of breast cancer at a later date. If this were true, it might follow that hormone dependency would vary among patients. This in turn would mean that measurement of a single hormone or its metabolites would be unlikely to provide an efficient means of prediction of response to therapy. For example, if 80% of the patients had tumors responsive to changes in the levels of the circulating estrogens (or on the presence or absence of receptor sites) and the remainder had tumors primarily dependent on pituitary stimuli, then there would be an inevitable degree of inaccuracy in any predictive system based on a single hormone. A complete assessment of endocrine function would be necessary and, while this is not technically impossible, the facilities required for the investigation of large groups of patients would be formidable.

Biochemical Investigations Versus Practical Prediction of Response

For the reasons listed, it seems quite likely that a definitive method for the prediction of response to endocrine therapy may be very difficult to achieve. None of the work so far published gives any hope of a clear-cut, universal difference between unresponsive and responsive patients. In any event, response is a continuous variable, and the best that can be expected is a method that will identify patients with a marked and prolonged response to therapy and those with a particularly poor prognosis. Possibly, in the future, an accurate predictor will be discovered; *e.g.,* the amount or even the presence or absence of an estradiol receptor site may be of crucial importance in determining whether or not a patient responds to a particular treatment. The likelihood is that partial correlations with response will be found, rather like that found for the discriminant function.

All work aimed at the discovery of such predictors has to begin with an investigation of endocrinological or biochemical differences between responsive and unresponsive patients, on a retrospective basis. Scores of these biochemical reports have appeared in the literature in the last 10 years. This work may be extremely valuable for the insight that is provided into the basic biology of the tumor and its host but, almost invariably, the next two logical steps in establishing the value of the predictor for routine use in the clinic are missing. These are a prospective test of the initial retrospective study, followed by the introduction of the test into clinical practice, and the demonstration that the response rate improves for those patients selected for treatment.

In the case of the discriminant function, the first and second stages of this process have been accomplished and approximately one-third of the patients with advanced breast cancer are no longer treated by endocrine ablation at Guy's Hospital, London, as a result of the steroid measurements. However, there is still no formal proof that the third condition for a useful predictor has been satisfied; *i.e.,* that patients who are treated by adrenalectomy or hypophysectomy, as a result of selection by the discriminant, have a higher remission rate than patients not so treated. Such evidence can only be obtained by still another prolonged clinical trial which is now in progress.

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Will the Best Model of Breast Cancer Please Come Forward?[1, 2, 3]

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PERHAPS I should have entitled this essay, "The Most Interesting Mammary Tumor I Have Known." I mean no disrespect or levity by these opening remarks, but rather, to set the tone for the problem that currently faces one investigator devoting his time to the study of mammary gland neoplasia. Several years ago, I presented some of our data on a rodent mammary adenocarcinoma at the Laurentian Hormone Conference *(1)*. Afterward, Dr. G. A. Overbeek asked me why we did what we did; *e.g.*, is there *any* experimental mammary tumor similar to human breast cancer, and if so, might it not be wiser to choose a totally different approach to the problem? I must admit that, because this query was posed near the end of the discussion period, it was rather easy to beg the question (as lunch was imminent) and, fortunately, Dr. S. Solomon brought the session to an end with the statement: "After 2 hours' discussion of an experimental breast tumor, it seems clear to me that if we don't look for similarities to human tumors, we won't find any." However, the question of why we are approaching this problem along the lines we have chosen has a gnawing quality that cannot be dismissed in a simple manner. I wish I could now state that we have the answer and here it is. Unfortunately, that is not the case. All that I can do is relate our most recent studies and the direction they are taking and, in so doing, offer you the opportunity to ponder some of the problems involved.

I will consider two general aspects of our work and put our data into some perspective with work by other investigators. We have been using the term "biochemical characterization" to describe much of this work. The data obtained under this very broad generalization are more limited than I really desire. One might call these studies of various biochemical parameters in different mammary tumors a hopeless effort to catalog a huge number of cell components of a neoplasm, with the unshakable faith that something unique will be uncovered in some particular neoplasm to distinguish it from all others. Such optimism may not be justified in light of the generalizations already proposed by Warburg *(2)* and Greenstein *(3)*, though it is now known that neoplasms can be found that possess unique properties not found in other related tumors, even though the tumors are similar morphologically *(4, 5)*. The investigator might expect to observe that there are quantitative differences rather than qualitative differences among tumors, and a survey of many different mammary carcinomas may reveal a degree of uniqueness that could be related to other parameters, such as growth rate and the influence on it by alterations of the hormonal milieu of the host.

Transplantable Rodent Mammary Tumors

A considerable body of knowledge can guide us in approaching the problem of breast cancer. If we wish to obtain an experimental model in a laboratory animal, we can characterize the tumor

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[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

[3] The studies performed in the author's laboratory were supported by Public Health Service contracts SA-43-PH-2395 and PH43-65-1050 from the Endocrine Evaluation Branch, General Laboratories and Clinics, National Cancer Institute.

[4] I acknowledge the excellent technical help of C. Bell, I. Michel, H. Goldenberg, and M. J. Carrington. The studies with 13,762 mammary tumors were done in collaboration with Dr. A. Segaloff, Ochsner Foundation, New Orleans, La.; the data on the DMBA- and MCA-induced carcinomas came from collaborative experiments with Drs. M. Gruenstein, D. M. Meranze, and M. B. Shimkin, Fels Research Institute, Temple University School of Medicine, Philadelphia, Penna.; the study of human breast cancer is a continuing collaborative program with Drs. R. A. Orlando and F. L. Archer, University of Chicago School of Medicine, Chicago, Ill. I am most grateful to Drs. J. L. Wittliff and T. C. Hall, University of Rochester School of Medicine and Dentistry, for their thoughtful and critical review of this manuscript and for their stimulating comments and discussion of these data.

by examining the effects of hormones on the most obvious measurable parameter, tumor growth. The investigator can categorize the particular tumor as hormone-dependent, hormone-responsive, or hormone-independent, terms used by Furth *(6)* to describe characteristics of endocrine-related neoplasms. This was the first question we asked when we began study of the R3230AC mammary adenocarcinoma of the Fischer rat, a slow-growing variant of a hormone-responsive neoplasm originally described by Dunning *(7)*. It was soon observed that tumor growth was not influenced by the sex of the host animal or by oophorectomy of the tumor-bearing rat, whereas growth of the carcinoma was significantly reduced after administration of estrogen or androgen to the animal bearing the tumor. It was also determined that the effect of treatment with estrogen was similar in either intact or oophorectomized hosts and also when hormone treatment was initiated immediately after tumor transplantation as well as when treatment was delayed for a time so as to allow the neoplasm to become established. Such was not the case for androgen therapy, since inhibition of neoplastic growth was no longer evident when androgen was administered to animals with well-established carcinomas. All these data suggested that the R3230AC neoplasm was hormone-responsive *(8)*.

In addition to the gross studies, the microscopic morphology of this neoplasm was studied and the effect of hormonal treatment upon the histologic appearance was evaluated. We found that estrogen, administered alone or in combination with prolactin, produced a marked secretory state in the epithelial cells of the carcinoma, accompanied by a considerable accumulation of a milk-like fluid. We concluded that the R3230AC carcinoma was capable of a lactation-like response and that the growth of the neoplasm was decreased in conjunction with the induction of a secretory state *(1, 8)*.

With these findings, it was logical to assume that many biochemical occurrences were being altered by administration of estrogen and that it would be profitable to examine certain selected parameters in neoplasms from animals that had received hormonal therapy. However, we had to decide which biochemical endpoints should be measured. We were guided by the literature pertinent to the biochemistry of the mammary gland and to the changes that occur during pregnancy and lactation *(9, 10)* and by the report of Rees and Huggins *(11)* on several nicotinamide adenine dinucleotide phosphate (NADP)-linked dehydrogenase enzymes in 3-methylcholanthrene (MCA)-induced mammary adenocarcinomas of the rat. Our initial investigations were devoted to the study of glucose-6-phosphate dehydrogenase, NADP-isocitrate dehydrogenase (decarboxylating), and NADP-malate dehydrogenase (decarboxylating)—enzymes that produce reduced NADP as a result of their catalytic action. This reduced pyridine nucleotide is essential for lipogenesis, protein synthesis, and steroidogenesis. We have investigated additional enzymes since and, again, our selection was based on the desire to learn about other aspects of carbohydrate, lipid, and amino acid metabolism and how administration of hormones might influence the activities of these enzymes. We thought certain tumors might exhibit biochemical patterns that could be correlated with morphologic changes in response to treatment with hormones. We learned that the inhibition of growth of the R3230AC tumor by administration of estrogen was accompanied by a totally different pattern of enzyme activities from the inhibition of tumor growth induced by the administration of testosterone propionate *(1)*.

While pursuing this work, the literature on hepatomas made it evident that different biochemical characteristics of microscopically similar neoplasms might be related to the growth rate of the tumor *(12–15)*. The most recent studies of hepatomas having normal karyotypes, but exhibiting significant differences in enzyme patterns and responses to environmental changes, added additional conviction to our efforts to establish biochemical characteristics of different mammary tumors *(16, 17)*. We embarked on a program to examine other experimental mammary tumors and selected the 13762 transplantable tumor, which had been originally induced by 7,12-dimethylbenz[*a*]anthracene (DMBA), and several of the sublines of this tumor *(18, 19)*. These sublines were developed by Segaloff by constant treatment of tumor-bearing hosts with exogenous hormones, resulting in neoplasms having different growth rates and microscopic morphology *(18)*. Our studies showed that alterations in enzyme activities and growth were manifested by simply transplanting the neoplasms, usually maintained in the female rat, to a male rat of the same strain. It was interesting that the alterations in enzyme activity profiles were in the direction of the tumor line as it appeared in the usual host. For example, the highest activities for several enzymes were found in the normal female tumor line maintained in the female host and the lowest activities of these enzymes were found in the normal male tumor line maintained in the male host. Transplantation of either tumor line into animals of the opposite sex produced tumors with biochemical profiles that were between the values of the two extremes, and the direction of the changes was exactly as anticipated from the usual values. The glucose-6-phosphate dehydrogenase activities/mg DNA were as follows: normal female tumor in females, 1.464; normal female tumor in males, 1.074; normal male tumor in females, 0.843; and normal male tumor in males, 0.476. The normal female tumor line was markedly inhibited by administration of androgen, and this was accompanied by significant decreases in most of the enzymes studied, an effect similar to that observed in the R3230AC tumor.

Carcinogen-Induced Rodent Mammary Tumors

Investigations were then initiated on the primary carcinogen-induced mammary neoplasms of the rat, it being reasoned that this type of tumor might more closely approximate the human disease. When we began these studies, a wealth of information had been acquired pertaining to methods of tumor induction by the carcinogens, the appearance and incidence of tumors relative to the dose and route of administration of the carcinogen, the histopathology of the resultant neoplasms, and the effects of hormones on the growth of these carcinomas *(20–27)*. It seemed most important that we initially examine the same biochemical parameters in mammary adenocarcinomas induced by DMBA or by MCA to ascertain similarities or differences of those primary neoplasms with the other transplantable tumors under study. It was known that most of the carcinogen-induced tumors were hormone-dependent and that the neoplasms would exhibit considerable regression subsequent to ablation of certain endocrine organs or to administration of certain hormones *(22, 24, 25, 27)*. In a recent summary of these data, we reported that DMBA-induced tumors arising after a single dose of the carcinogen showed differences in the activities of specific enzymes as compared to the activities of these enzymes in DMBA-induced tumors arising after multiple doses of the carcinogen *(28)*. Further, it was interesting to find that certain biochemical parameters in the MCA-induced tumors (induction of mammary neoplasms by MCA is accomplished only by multiple doses of the carcinogen) resembled more closely those of the DMBA-induced tumors which occurred after multiple doses of DMBA. Thus, a change in the regimen of feeding the carcinogen produced neoplasms with somewhat different biochemical profiles, an observation closely resembling the findings reported for carcinogen-induced hepatomas. These results suggest that many biochemical models of DMBA-induced mammary carcinomas may exist, each having differences in growth and responsiveness to therapeutic regimens. It is certain that the experiments reported only begin to characterize biochemically the available experimental mammary tumors and that considerable areas remain for future research.

Human Breast Cancer

The premise of our work was that animal models of the human disease do exist. Simply, *all* one needs to do is to obtain or construct the disease model, prove it is valid, dedicate oneself to work on the model to understand it, find agents or procedures that affect or cure the model, and confidently apply these data to predicting the outcome of their application to the clinical disease. The foregoing is the story line of many scientific sagas, most of them necessitating a causative agent of the disease. Since many agents are potentially causative of human cancer, *e.g.*, viruses, chemicals, hormones, and X ray, we cannot limit our studies to a definition of the cause to build a proper model. Instead, retrospective studies could be conducted with human breast cancer to define the characteristics of the human neoplasms. Such a study would tell us what metabolic similarities exist between human and rodent tumors, which of these parameters has been altered from normal breast tissue, and, perhaps, what biochemical alterations are associated with frank carcinoma or benign proliferative processes. The literature contains little information on the biochemistry of human breast cancer *(29)*, and it seemed essential that the human disease be studied to establish a guideline for the selection of a valid animal model. Our studies of human breast cancer, which were limited by design to infiltrating ductal carcinoma, normal mammary tissue, and fibrocystic disease of the breast (a proliferative but benign disease), have revealed some very interesting data. Compared to normal breast, the activities of pyruvate kinase, glucose-6-phosphate dehydrogenase, NADP-isocitrate dehydrogenase, phosphoglucomutase, glutamate dehydrogenase, and hexokinase in the carcinomas were elevated 10–30-fold, when enzyme activity was expressed per 100 mg of tissue weight, or from 2–8-fold, when enzyme activity was expressed per mg of DNA ($P < 0.001$). RNA and DNA levels were increased in neoplastic tissues as well as cholesterol and free fatty acids, whereas α-glycerolphosphate dehydrogenase activity and triglyceride content were significantly decreased in the tumors. Samples of fibrocystic disease exhibited an increase in phosphoglucomutase activity/mg DNA, the other parameters being similar to normal breast tissue. From the measurements made, it is now possible to grade the tissues by biochemical properties in the following order: normal = fibrocystic disease <carcinoma *(30–32)*. This is a most important distinction, since it implies that there are changes that are unique to carcinoma and these alterations are not present in a benign disease, which possesses proliferative capacities.

Comparison of Models

With these data we can compare the biochemical data in human breast carcinomas with the data obtained in several rodent neoplasms. The data, summarized in table 1, include human infiltrating ductal carcinomas, DMBA- and MCA-induced carcinomas, the transplantable R3230AC tumor, and the normal male and female lines of the 13762 carcinoma. The expression of enzyme activity in this table is on the basis of tissue weight, allowing for an easy comparison. The enzyme activities are presented in a decreasing order of magnitude found in the human tumors, with pyruvate kinase demonstrating the highest activity and glucokinase the lowest activity. Certain of the enzymes show similar activities in the experimental rodent carcinomas when compared with the human tumors. Table 1

TABLE 1.—Enzyme activities, nucleic acids, and lipids in human breast carcinomas and several rodent mammary tumors *

Measurement †	Human IDC	DMBA	MCA	R3230AC	13762F	13762M	HMC
Enzyme:							
PYK	7.52‡	8.88		9.16			
GPI	4.05	2.32	2.67	4.58	4.47	2.46	2.53
AAT	0.422	1.81		1.25			
ICD	0.304	0.517	0.564	0.380	0.521	0.325	0.148
G6PD	0.134	0.277	0.494	0.414	0.449	0.179	0.882
α-GPD	0.049	0.128	0.125	0.059	0.094	0.055	0.011
PGM	0.044	0.230	0.271	0.241	0.399	0.562	0.065
GDH	0.035	0.029	0.030	0.033	0.036	0.014	
HK	0.031	0.034		0.059			
ME	0.028	0.105	0.110	0.512	0.181	0.028	0.053
GK	0.002	0.002		0.004			
Nucleic acid:							
RNA	4.67	4.26	3.40	6.23	8.87	4.05	7.36
DNA	6.46	7.77	8.07	6.55	4.74	2.79	6.80
RNA/DNA	0.77	0.56	0.43	0.90	1.89	1.44	1.06
Lipid:							
CHOL	2.39	2.28	2.76	1.64			
FFA	3.10	2.29	3.35	9.59			
TG	49.4	28.1	7.6	25.2			

* Tumors studied and the number of specimens, shown in parentheses below, were: IDC, human infiltrating ductal carcinoma of the breast (35); DMBA, tumors induced by a single, 15-mg intubation of DMBA (24); MCA, tumors induced by intubation of 90 mg MCA, 10 mg/week for 9 weeks (22); R3230AC, transplantable tumor (45); 13762F, DMBA-induced transplantable tumor, normal female line (10); 13762M, DMBA-induced transplantable tumor, normal male line (10); and HMC, MCA-induced transplantable tumor (12).

† Abbreviations for biochemical parameters are: PYK, pyruvate kinase; GPI, glucosephosphate isomerase; AAT, aspartate aminotransferase; ICD, NADP-isocitrate dehydrogenase, decarboxylating; G6PD, glucose-6-phosphate dehydrogenase; α-GPD, α-glycerolphosphate dehydrogenase; PGM, phosphoglucomutase; GDH, glutamate dehydrogenase; HK, hexokinase; ME, NADP-malate dehydrogenase, decarboxylating; GK, glucokinase; CHOL, cholesterol; FFA, free fatty acids; and TG, triglycerides.

‡ Enzyme activity expressed as μmoles of pyridine nucleotide altered/minute/100 mg tissue weight; nucleic acids and lipids expressed as μg/mg of tissue weight.

also shows the levels of nucleic acids in the neoplasms. The RNA/DNA ratio of the R3230AC tumor appears quite similar to the ratio of nucleic acids in the human neoplasms. Assay of various types of lipids in these tumors revealed certain similarities. For example, the cholesterol levels of the human and DMBA-induced tumors were the same, whereas the free fatty-acid contents of the MCA-induced tumors were most similar to the free fatty acids in the human carcinomas.

Because of the differences in DNA in these tumors and the assumption that such differences may reflect cell number rather than polyploidy, it is advisable to express enzyme activity in terms other than tissue weight. One such approach, which we have used, is to calculate enzyme activity per mg of DNA. The results of these calculations are presented in table 2. Again, similarities in the activities of some enzymes were quite apparent, when comparing the human carcinomas with the animal tumors, such as the activities of NADP-isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, α-glycerolphosphate dehydrogenase, and NADP-malate dehydrogenase in the DMBA-induced tumors and infiltrating ductal carcinomas. These data also demonstrate that the activities of aspartate aminotransferase and phosphoglucomutase are considerably lower in human tumors than in rodent neoplasms. It would appear that the primary DMBA-induced rodent tumor has several metabolic characteristics in common with human breast cancer *(31)*.

TABLE 2.—Activities of enzymes per mg DNA in human breast carcinomas and several rodent mammary tumors

Enzyme*	Human IDC	DMBA	MCA	R3230AC	13762F	13762M	HMC
PYK	14.86	9.84		12.81			
GPI	6.61	2.30	3.35	5.47	9.99	6.16	3.74
AAT	0.667	2.45		1.80			0.467
ICD	0.493	0.577	0.721	0.628	1.105	0.823	0.217
G6PD	0.212	0.351	0.617	0.625	1.464	0.476	1.300
α-GPD	0.105	0.155	0.127	0.100	0.154	0.036	0.016
PGM	0.070	0.423	0.354	0.377	0.460	0.154	0.096
GDH	0.048	0.036	0.039	0.044			0.010
HK	0.058	0.030		0.083			
ME	0.044	0.063	0.122	0.753	0.443	0.104	0.077
GK	0.004			0.006			

* Abbreviations used are the same as in table 1. Enzyme activity expressed as μmoles cofactor/minute/mg DNA.

There are other ways of looking at the same data to gain additional insight into their significance. One way is to compare the activities of the various enzymes relative to a common point; *i.e.,* set one enzyme activity equal to 100 and express the other enzyme activities in terms of this arbitrary standard. Such an approach was employed by Shonk and Boxer in examining human and rodent tumors *(29)*. Table 3 summarizes these calculations, in which the activity of glucose-6-phosphate dehydrogenase was set equal to 100 and all the other enzyme activities/mg DNA were compared to it. One could interpret the data in table 3 as indicating that pyruvate kinase activity, which has already been shown to be elevated on a tissue weight basis and on a per-mg DNA basis, is disproportionately elevated in the carcinoma with respect to normal breast tissue. However, aspartate aminotransferase, phosphoglucomutase, glutamate dehydrogenase, and hexokinase activities retain their same relationship to the activity of glucose-6-phosphate dehydrogenase in both normal and neoplastic breast tissues. In contrast, the data from the rodent carcinomas show somewhat different relationships. Most of the rodent tumors demonstrate decreased NADP-isocitrate dehydrogenase, glucosephosphate isomerase, aspartate aminotransferase, and NADP-malate dehydrogenase activities compared to normal rat breast tissue. All the neoplasms demonstrated a striking decrease in α-glycerolphosphate dehydrogenase activity relative to the normal breast. These data are helpful in pointing out those metabolic steps that are disproportionately affected in the neoplasm.

It is of interest that one can find somewhat similar relationships existing in the normal mammary gland when it is examined during pregnancy and lactation. Enzyme activities, as well as protein, nucleic acids, and levels of pyridine nucleotides, in mammary glands were thoroughly evaluated by Baldwin and his colleagues *(9, 33, 34)*. Using some of their data, I calculated the ratios of the

Table 3.—Relative enzyme patterns in human and rodent breast tissues

Enzyme*	Human IDC	Human normal	DMBA	MCA	R3230AC	13762F	13762M	Rodent normal
PYK	7007	1653	2805		2050			1670
GPI	3117	4089	655	543	875	683	1293	1845
AAT	315	387	699		288			1292
ICD	233	162	164	117	101	76	173	316
G6PD	100	100	100	100	100	100	100	100
α-GPD	50	1843	44	21	16	11	8	1549
PGM	33	20	121	57	60	31	32	108
GDH	23	18	10	6	7			20
HK	27	24	9		13			100
ME	21	36	18	20	121	30	22	272
GK	2	44			1			67

* Abbreviations used are the same as in table 1. Enzyme activities used for these calculations, setting G6PD = 100, were on the basis of μmoles cofactor/minute/mg DNA.

activities of several enzymes, setting glucose-6-phosphate dehydrogenase equal to 100. The activities for the mammary gland on day 20 of pregnancy are as follows: glucose-6-phosphate dehydrogenase, 100; glucosephosphate isomerase, 347; aspartate aminotransferase, 215; NADP-isocitrate dehydrogenase, 103; phosphoglucomutase, 29; α-glycerolphosphate dehydrogenase, 21; and hexokinase, 17. These data are based on enzyme activity per mg of tissue. If we examine the interrelations of the same enzymes in human and rodent tumors, using our data calculated on a wet tissue weight basis, we can see some similarities (table 4). For example, aspartate aminotransferase activity is about three times higher than glucose-6-phosphate dehydrogenase activity in human tumors and in the R3230AC tumor, compared with the twofold increase seen in the pregnant mammary gland. It is also interesting that α-glycerolphosphate dehydrogenase activity for all the tumors is considerably reduced relative to glucose-6-phosphate dehydrogenase, a pattern similar to the pregnant mammary gland. The same is true for hexokinase. Phosphoglucomutase activity, in this relative calculation, is quite comparable in the human tumors and the pregnant rat mammary gland. Depending on the baseline used for control tissue, neoplasms may resemble the pregnant mammary gland in certain respects, and this leads to some difficulty in defining essential neoplastic changes in the breast. For example, most neoplasms demonstrate low levels of α-glycerolphosphate dehydrogenase, but so does the pregnant mammary gland. Can this enzyme disfunction be a *sine qua non* of neoplasia?

It is apparent from the data presented that the deviation of neoplastic tissues from normal tissues is in the same direction for most of the parameters measured in both human and rodent neoplasms. The major difference among the various tumors is quantitative rather than qualitative. Thus, for any one parameter, *e.g.*, NADP-isocitrate dehydrogenase, phosphoglucomutase, or glutamate dehydrogenase, the deviation from the normal breast

Table 4.—Relative enzyme patterns in human and rodent mammary carcinomas

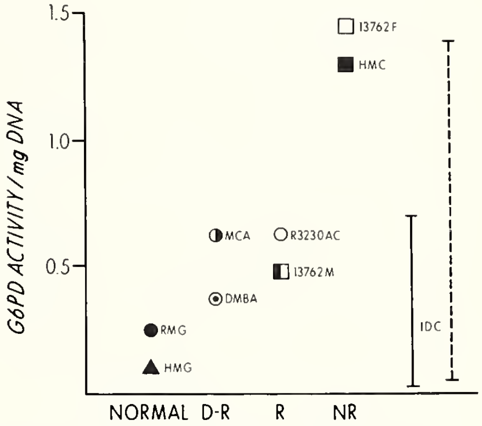
Enzyme*	Human IDC	Human normal breast	DMBA	MCA	R3230AC	13762F	13762M	Rodent normal breast
PYK	5199	1989	3205		2212			469
GPI	3083	5055	838	540	1106	996	1374	3819
AAT	322	500	652		301			419
ICD	227	233	187	114	92	116	182	313
G6PD	100	100	100	100	100	100	100	100
α-GPD	47	1522	46	25	14	21	31	1744
PGM	33	22	83	55	58	89	314	175
GDH	23	22	10	6	8	8	8	19
HK	31	33	12		14			31
GK	2	22	1		1			19
ME	21	44	38	22	124	40	16	188

* Abbreviations used are the same as in table 1. Enzyme activities used for these calculations, setting G6PD = 100, were on the basis of μmoles cofactor/minute/100 mg tissue weight.

tissue was comparable. In general, rodent tumors have a wider range in their deviations of RNA and DNA levels and considerably less free fatty acids and triglycerides. The difference in nucleic acids may be a reflection of differences in growth rates, turnover rates, etc., whereas the differences in lipid contents may be due to the presence of normal adipose tissue in the human tumors and in some of the primary carcinogen-induced tumors, as contrasted to the transplantable tumors.

This brings me to a speculation of the significance and meaning of these data and their applicability to the clinical disease. Conceivably, some of the parameters measured will fit into a pattern that will be predictive of hormone-responsive tumors, whereas another biochemical pattern may emerge for neoplasms that are not responsive to hormonal therapy. Such patterns have been reported for MCA-induced mammary tumors by Rees and Huggins *(11)*, and, recently, King *(35)* suggested that hormone-dependent tumors may demonstrate lower activities of the enzymes associated with anaerobic glycolysis.

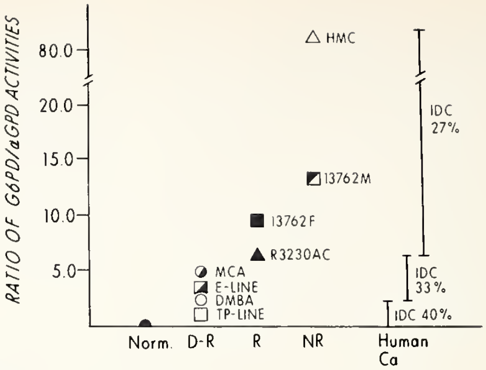
Text-figure 1 summarizes our data on glucose-6-phosphate dehydrogenase activity in various rodent tumors and in normal mammary glands. There appears to be a correlation between enzyme activity and the ability of the rodent neoplasm to respond to hormonal treatment. The lowest enzyme activities were found in the DMBA- and MCA-induced primary carcinomas, which are both hormone-dependent (D) and hormone-responsive (R). An intermediate level of enzyme activity was found in the R3230AC and the 13762M neoplasms, two tumors that are hormone-responsive (R) but not hormone-dependent. The highest glucose-6-phosphate dehydrogenase activities were observed in the HMC tumor and in the 13762F tumor, the former neoplasm being unresponsive to any hormonal treatment and the latter neoplasm being unresponsive to estrogen therapy, and neither of these tumors was responsive to oophorectomy. Also shown in this text-figure is the range of enzyme activity found in 50 human infiltrating ductal carcinomas of the breast *(solid line)* and the activity of normal human mammary glands. Let us assume that human breast cancer will deviate from normal breast tissue in a manner similar to the way rodent neoplasms differ from rodent breast tissue, *i.e.*, comparing on a relative scale the human tumors. We see that the adjusted range of enzyme activity *(dashed line)* for human carcinomas encompasses the full range of rodent tumors studied. Thus, if data from experimental tumors are translatable to the clinical disease, the glucose-6-phosphate dehydrogenase activity of human tumors examined to date fits into the same range of activities of the rodent neoplasms being studied. Obviously, we now need to determine the course of the disease in those patients from whom we have performed a biochemical assay on their carcinomas. Such a study could supply the data to establish a predictive test for therapeutic responsiveness.



Text-figure 1.—Glucose-6-phosphate dehydrogenase activity (μmoles NADPH produced/minute/mg DNA) of several rodent mammary tumors, human infiltrating ductal carcinoma of the breast, and normal mammary glands of the rat (RMG) and of humans (HMG). *Solid vertical line* represents the overall range of enzyme activity in human carcinomas, and *dashed vertical line* represents the adjusted range of enzyme activity in human tumors, the values being obtained by normalizing the human breast tissue to rat breast tissue. The abbreviations used are: D, hormone-dependent; R, hormone-responsive; and NR, nonresponsive to hormonal therapy.

The data obtained from study of rodent tumors also form the basis for a postulation as to why estrogen treatment may inhibit some tumors and not alter the growth of other neoplasms. We have shown that several of the rodent carcinomas respond to administration of estrogen with an increase in the activity of glucose-6-phosphate dehydrogenase and a decrease in tumor growth. Perhaps it is fortuitous that the maximum enzyme activities we have achieved in these neoplasms by injection of estrogen is in the order of 1.0–1.4 units of activity per mg DNA, a level of activity identical to that found in the nonresponsive tumors. This high level of activity for this enzyme may be the maximum for neoplastic breast tissue, and only if the enzyme is below this level of activity can we expect estrogen treatment to induce a response. On the other hand, removal of the ovaries or administration of androgens results in a decrease in the activity of glucose-6-phosphate dehydrogenase in hormone-dependent or hormone-responsive tumors, respectively. Let us assume that a critical level of enzyme activity is required for the metabolic economy of the neoplastic cell and no effect on tumor growth will be observed unless the enzyme is decreased below this critical level. Since we cannot decrease the activity of this enzyme by administration of estrogens, we must approach this situation with other agents, *e.g.,* androgens. We have found that the 13762F neoplasm significantly decreases in growth after administration of androgens, a response that is accompanied by a 50% decrease in glucose-6-phosphate dehydrogenase activity. The glucose-6-phosphate dehydrogenase activity in the HMC tumor is unaffected by androgen treatment, and tumor growth is not impeded. It would appear that the control of glucose-6-phosphate dehydrogenase activity represents a most important aspect of the metabolism of the neoplastic cell, and alteration of the activity of this enzyme may be indicative of responsiveness of the tumor.

Another enzyme that appears to be correlated with responsiveness is α-glycerolphosphate dehydrogenase. In this instance, the relationship is an inverse one, in which higher enzyme activities were found in dependent tumors and very low levels of enzyme were observed in the nonresponsive tumors. This relationship may represent an estimation of the degree of deviation from normal, since the levels of α-glycerolphosphate dehydrogenase are very high in normal breast tissue. It is most interesting that regression of the DMBA-induced carcinoma is accompanied by an increase in the activity of this enzyme. Thus, it is proposed that a low ratio of glucose-6-phosphate dehydrogenase to α-glycerolphosphate dehydrogenase may be indicative of hormone-dependent carcinomas and, as the ratio increases, the hormone responsiveness decreases (text-fig. 2).



Text-figure 2.—Relationship of glucose-6-phosphate dehydrogenase activity to the activity of α-glycerolphosphate dehydrogenase in rodent and human mammary carcinomas. The rodent tumors examined were: TP-line, a subline of the 13762 transplantable tumor which is under constant treatment with testosterone propionate and has become dependent on the hormone for growth; DMBA, carcinogen-induced primary neoplasm which is hormone-dependent and hormone-responsive; E-line, a subline of the 13762 transplantable tumor which is under constant treatment with estradiol and is dependent on the hormone for growth; MCA, carcinogen-induced primary neoplasm which is hormone-dependent and hormone-responsive; R3230AC, transplantable tumor which is not hormone-dependent but is hormone-responsive; 13762F, transplantable tumor maintained in female rats which is not hormone-dependent but is hormone-responsive; 13762M, transplantable tumor maintained in male rats which is neither hormone-dependent nor hormone-responsive; and HMC, transplantable tumor which is neither hormone-dependent nor hormone-responsive. The human infiltrating ductal carcinomas of the breast demonstrate a complete range of ratios of enzyme activities, and the percentages given represent the number of tumors found to fall in the ranges shown. Other abbreviations used are the same as in text-figure 1.

Additional Biochemical Properties of Rodent Models

Initially, I indicated that this discussion would cover a second aspect and that it would deal with studies of certain tumor models. The R3230AC carcinoma of the Fischer rat has some unique properties that make it a most interesting neoplasm for examination of basic biochemical mechanisms of hormone action. Our early finding of a striking secretory response in the neoplasm after treatment with estrogen stimulated further study of the biochemical events associated with this morphologic alteration. We examined the time course of the response by obtaining neoplasms at frequent intervals after administration of a single dose of estrogen. It was learned that a maximum increase in glucose-6-phosphate dehydrogenase and NADP-malate dehydrogenase occurred within 2–4 days after hormone administration, after which the activities of these enzymes returned to the control of nonstimulated levels. With this test system, it was possible to demonstrate that concomitant administration of actinomycin D or cycloheximide prevented the estrogen-induced increase in enzyme activity as well as the incorporation of labeled precursors into RNA and protein *(36, 37)*. We concluded that the R3230AC carcinoma had retained a degree of differentiation that allowed the hormone to initiate a series of functional events culminating in the production of a milklike substance. Analyses of this milklike fluid revealed that it contained lactose and proteins, which resembled the electrophoretic properties of casein and whey proteins of rat milk *(38)*.

Several investigators have examined the R3230AC tumor for metabolic similarities to lactating tissue. Shatton *et al. (39)* found that the milklike fluid of the neoplasm contained lactose at a concentration about 1–2% of that found in rat milk, a result agreeing well with our earlier finding. However, these investigators did not find an elevation in the activities of galactose epimerase or uridine diphosphate (UDP)-glucose pyrophosphorylase, two enzymes that are involved in the production of lactose. Although they found that the activities of the enzymes in the R3230AC carcinoma were higher than those in normal mammary glands, the activities of the enzymes were considerably lower than those observed in the lactating mammary gland. This result could explain the low lactose content observed in the milklike secretion of the tumor.

Recently, McGuire *(40)* reported that the R3230AC carcinoma possesses significant quantities of lactose synthetase, when the tumor is in virgin female rats. The administration of prolactin to tumor-bearing rats induces an increase in the activity of this enzyme to levels found only in mammary glands of late pregnancy or during lactation. This is a most intriguing finding, since lactose synthetase is composed of two proteins *(41)*: The A protein, which has galactosyltransferase activity and catalyzes the synthesis of *N*-acetyllactosamine from UDP-galactose and *N*-acetylglucosamine, and the B protein, which is α-lactalbumin and which apparently behaves as a modifier of the A protein, resulting in an alteration of the substrate specificity of the A protein from acetylglucosamine to glucose, so that lactose synthesis proceeds. This rather complicated system is apparently operative in the transplantable neoplasm, a rather remarkable occurrence when considering the number of years that the neoplasm has been maintained by transplantation. The finding of McGuire also strengthens the thesis that neoplasms may retain highly specialized functions that could be controlled by endogenous or exogenous factors, *i.e.*, hormones. These findings and those of our earlier studies, which showed that prolactin inhibited tumor growth when administered with estrogen *(42)* or alone via a prolactin-secreting mammotrophic tumor *(43)*, strengthen our conclusion that certain hormones may be directing substrates toward nonutilizable end products and, in the process, direct the energy of the neoplasm away from growth. The R3230AC tumor may be an unusual mammary carcinoma and may not be representative of all human breast cancer. However, some human tumors may have retained the capacity for specialized cellular function and recognition of this property may be very useful in planning a therapeutic regimen.

Turkington and I *(44)* also examined the R3230AC tumor by utilizing organ explant methods. We reported that the neoplasm did not require insulin to initiate DNA synthesis as does the normal (mid-pregnant) mammary gland of the Fischer rat. These data suggest that the neoplasm is differentiated and therefore capable of responding to other hormonal stimuli necessary to initiate milk production. Turkington and Riddle *(45)* demonstrated that estradiol stimulated lactose synthetase activity and casein production threefold to fourfold in the R3230AC carcinoma cultured *in vitro*. In contrast, Heuson and Legros *(46)* showed that explants of the DMBA-induced mammary adenocarcinoma were stimulated by insulin *in vitro*, as measured by the increase in thymidine incorporation into DNA. Mioduszewska *et al. (47)* have conducted experiments with human mammary carcinomas in explant organ culture and have investigated the effects of hormones on cell growth *in vitro*. They concluded that patients with tumors, which displayed a stimulation to prolactin, had the most favorable prognosis for survival. All of these results imply that the extent of differentiation of a neoplasm may be an important characteristic in connection with the hormone dependency or hormone responsiveness of the carcinoma. Thus, information obtained from the study of the mechanisms of differentiation of mammary glands may have direct application to designing therapeutic regimens.

Lest one think that the R3230AC carcinoma is the only experimental model that is of interest, I should point out that the DMBA-induced mammary adenocarcinoma is a model that has great potential. Since it is a primary neoplasm, it has a mixed cell population, a situation that causes most biochemists to shake their heads and turn to transplantable tumors of more uniform cell populations. However, the more heterogeneous situation regarding cellular constituency is common in human breast cancer and, as such, the DMBA-induced neoplasm may represent a closer counterpart of the clinical disease. It is noteworthy that a percentage of these carcinogen-induced tumors lactate spontaneously *(48)* and our studies demonstrated that the administration of estrogen will induce a secretory response in these neoplasms, accompanied by changes in enzyme activities similar to those seen in the R3230AC tumor *(28)*. Further, we demonstrated significant decreases in pyruvate kinase, glucose-6-phosphate dehydrogenase, NADP-malate dehydrogenase, and phosphoglucomutase activities in DMBA-induced carcinomas within 5 days after oophorectomy, a time at which significant changes in tumor size have not yet occurred *(49)*.

Recently, Beuving *et al. (50, 51)* presented data indicating that DMBA could induce hyperplastic nodules, which, upon transplantation to the gland-free fat pad of isologous rats, grew substantially but did not develop into carcinomas. Unlike the situation with the mouse, the question is still unanswered as to whether these carcinogen-induced hyperplastic nodules are preneoplastic. This is a most interesting area for future work, since it is quite important to determine the biochemical characteristics of a preneoplastic lesion. Such studies have been performed by Kopelovich *et al. (52, 53)* with certain mouse adenocarcinomas and their preneoplastic alveolar nodules. Their findings indicated that the hyperplastic nodule resembled the pregnant mammary gland profile of enzyme activities rather than that seen in the adenocarcinoma. It will be of interest to learn if the same relationship holds for the DMBA-induced nodule.

SUMMARY

In summary, the questions that we have posed and the data that are being accumulated and examined for answers to these problems are as follows:

1) What biochemical properties are unique to breast cancer cells? Accumulated data allow us to identify with a good degree of certainty carcinomatous tissue of the breast on the basis of its biochemical characteristics as compared to fibrocystic disease of the breast or normal breast tissue. Additional studies to include fibroadenomas will provide additional data for establishing the parameters distinguishing malignant disease from benign proliferative processes.

2) Do any animal tumors resemble human breast cancer? Biochemical characterization of several rodent carcinomas and human infiltrating ductal carcinomas of the breast has provided data demonstrating certain metabolic similarities between the DMBA-induced carcinoma and human breast cancer. Data obtained from valid models should be more readily translated to the clinical disease, and the mechanism of action of agents would be better defined by use of valid models.

3) Can hormone-responsive tumors be identified? Data on glucose-6-phosphate dehydrogenase and α-glycerolphosphate dehydrogenase activities indicate that these enzymes show differences in hormone-dependent, hormone-responsive, and estrogen-unresponsive rodent neoplasms. Extension of these studies to human breast cancer accompanied by follow-up of the patient's course of disease should tell us if these relationships hold for the clinical disease and can be used for a predictive test.

4) Do any biochemical changes occur in a neoplasm soon after initiation of treatment? Data on hormone-dependent and hormone-responsive rodent carcinomas suggest that many significant alterations in enzyme activities occur preceding any significant changes in tumor growth. Extension of these studies to human tumors may provide a rapid method to predict the outcome of a selected therapeutic regimen.

5) Can we identify breast cancers that are responsive to nonhormonal agents? Studies with rodent and human breast tumors to identify the neoplasms' ability to concentrate and/or metabolize certain antimetabolites are being initiated, along

lines that have been used with success for leukemic cells.

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Estrogen Receptors and Breast Cancer Response to Adrenalectomy[1, 2, 3]

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BINDING OF ESTROGENS IN TARGET TISSUES

It has long been recognized that certain mammalian tissues, associated with the reproductive process, cannot grow and function optimally without minute amounts of steroidal compounds known as sex hormones. Although the actual basis of this hormone dependency remains obscure, an important difference between dependent and nondependent tissues was demonstrated by studies of the fate in various tissues of tritiated estrogens administered to hormone-deprived animals. The "target" tissues, such as uterus, vagina, and anterior pituitary, possessed a striking affinity for estradiol, indicating that they contain specific estrogen-binding components, now called "estrogen receptors" or "estrophiles."

Extensive investigations[5] on the incorporation and retention of radioactive steroid after the administration of physiologic amounts of various estrogens have established the principal characteristics of the interaction of estrogenic hormones with responsive tissues *in vivo*. In the rat, the target tissues show a striking affinity for estradiol, 17α-methylestradiol, 17α-ethynylestradiol, and hexestrol and a transient affinity for estriol. Each of these substances exerts its hormonal effect without undergoing chemical alteration, even though each is subject to extensive metabolic transformation elsewhere in the animal. In contrast, neither estrone nor mestranol binds to target tissues; the estrogenic action of these substances appears to depend on their metabolic conversion to estradiol and 17α-ethynylestradiol, respectively, which then accumulate in the target organs.

Specific incorporation of estrogens into hormone-dependent tissues is blocked by certain antiuterotrophic compounds, such as ethamoxytriphetol (MER-25), nafoxidine (Upjohn 11,100), Parke-Davis CI-628, and clomiphene—substances which do not affect the low level of estradiol incorporation into nontarget tissues. The inhibition of estradiol-induced uterine growth by various doses of nafoxidine was found to parallel reduction of estradiol uptake, which provided evidence that the estrogen-binding phenomenon is closely involved in the biological action of the hormone. In contrast, actinomycin D (AD) and puromycin, both of which block overall uterine growth as well as many of the early biochemical responses of the uterus to estrogen, do not interfere with the binding of estradiol to target tissues, which indicates that the estrogen-receptor interaction is an early step in the uterotrophic process, preceding the acceleration of biosynthetic reactions sensitive to AD or puromycin.

When surviving uterine tissue is exposed to dilute solutions of tritiated estradiol in Krebs-Ringer buffer at physiologic temperature, an interaction of hormone with receptor takes place, which shows all the characteristics of the *in vivo* phenomenon. This interaction *in vitro* does not require oxygen or added nutrients. As illustrated in text-figure 1, uterine tissue shows a striking incorporation of radioactive steroid, which, in the presence of specific binding inhibitors, such as nafoxidine or Parke-Davis CI-628, is reduced to the low level of

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[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

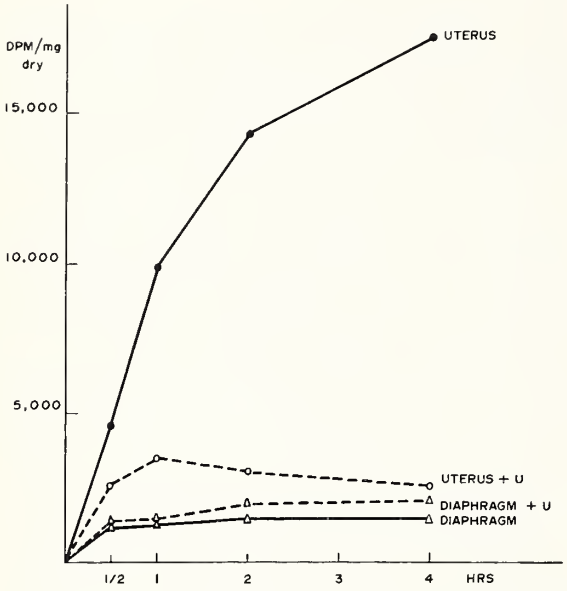
[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

[3] The human tumor studies were supported by Public Health Service contract PH 43–66–945 from the National Cancer Institute.

[4] We gratefully acknowledge the technical assistance of Marsha Gage, Suzie Adler, and Lois Kahn and the cooperation of Drs. Donald Ferguson, Paul Harper, Hilger Jenkins, René Menguy, Edward Paloyan, and John Prohaska of the University of Chicago Clinics and Dr. Frances Knock of Augustana Hospital in supplying clinical specimens.

[5] Review articles *(1–4)* give a more detailed description of these studies of estrogen binding in whole tissues, with specific reference to the contributions of Glascock, Stone, Martin, Baggett, Gorski, Talwar, King, Mahesh, Michael, Eisenfeld, Terenius, Callantine, Villee, and their associates.

nonspecific binding seen in diaphragm and other nontarget tissues. Thus, the uptake of estradiol in an *in vitro* system and its sensitivity to the action of specific inhibitors provide a simple means of distinguishing between estrogen-responsive tissues, which contain receptor proteins, and non-estrogen-responsive tissues, which do not.



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TEXT-FIGURE 1.—Concentration of radioactivity in dried, slit uterine horns and hemidiaphragms of immature rats after stirring for various times in 0.1 nM estradiol-6,7-^{3}H at 38°C in Krebs-Ringer-Henseleit glucose buffer (*p*H 7.3) in the presence and absence of 10 μM nafoxidine (Upjohn 11,100). Each *point* is the median value of 5 horns.

The foregoing studies of the fate of physiologic amounts of estrogenic hormones in whole tissues, both *in vivo* and *in vitro,* provided a basis for evaluating the significance of estrogen-binding phenomena in broken cell systems. As more detailed investigations were carried out,[6] it became apparent that the interaction of estradiol with uterine and other target tissues is not a simple association of the hormone with a single intracellular binding site but involves a more complex kind of phenomenon.

From a variety of evidence, summarized completely in *(4),* the interaction of estradiol with uterine tissue has been shown to take place by a two-step process (text-fig. 2). Estradiol first combines with an extranuclear "uptake receptor," a protein which, in sucrose density gradients of low ionic strength, sediments as some kind of aggregate (about 8S) but which dissociates into well-defined 4S binding units at higher ionic strength. This association with the hormone endows the receptor protein with the ability to undergo a temperature-dependent conversion from the 4S to a 5S form which accompanies migration of the complex into the nucleus. Most of the uterine estradiol becomes localized in the nucleus, from which it can be extracted as 5S complex by 0.3 M or 0.4 M KCl. Whether the 4S-to-5S transformation takes place within the nucleus (text-fig. 2A) or whether it precedes transfer to the nucleus (text-fig. 2B) cannot as yet be decided with certainty.

At present, the relation is not clear between the transformation sequence of receptor complexes and the stimulation of biosynthetic processes in the hormone-dependent tissue, although the same general type of two-step mechanism has recently been demonstrated for the interaction of several classes of steroid hormones with their target tissues *(4).* Possibly, the extranuclear protein is only a means of transporting the hormone into the nucleus, where it elicits its response by a mechanism independent of the receptor substance. On the other hand, the receptor protein itself may be the active agent, perhaps a participant in some nuclear process in analogy to the σ factor in microbial RNA polymerase systems. In hormone-dependent mammalian tissues, such a protein, synthesized in the ribosomes, may require activation by the hormone to reach its site of action in the nucleus. In any case, a principal role of the estrogen is to associate with the extranuclear receptor protein and to render this protein susceptible to temperature-dependent transformation and migration to the nucleus.

Unlike the 5S nuclear complex, which depends on the cytosol protein for its formation, the extranuclear 8S complex, or its 4S subunit, can be produced directly, even in the cold, simply by the addition of estradiol to the supernatant fraction of a uterine homogenate (text-fig. 3). If an inhibitor of specific binding, such as Upjohn 11,100 or Parke-

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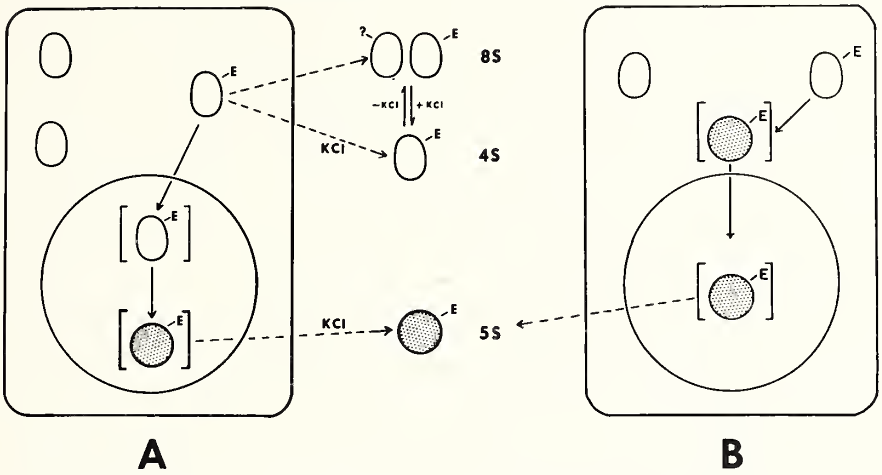
[6] See *(3–7)* for a more complete description of studies of the receptor complexes and their interaction, with specific reference to the contributions of Gorski, Talwar, Erdos, Baulieu, King, Korenman, Wotiz, Maurer, Stumpf, Bresciani, Attramadal, and their associates.

Davis CI-628, is also present, no hormone-receptor complex is evident. After sufficient tritiated estradiol is added to saturate the cytosol receptor, the amount of this protein can be estimated conveniently from the area of the 8S sedimentation peak. The excess radioactive hormone either will appear at the top of the tube (text-fig. 3A) or, if serum proteins are present, it will appear bound in weak association with albumin which sediments at 4.6S (text-fig. 3B).

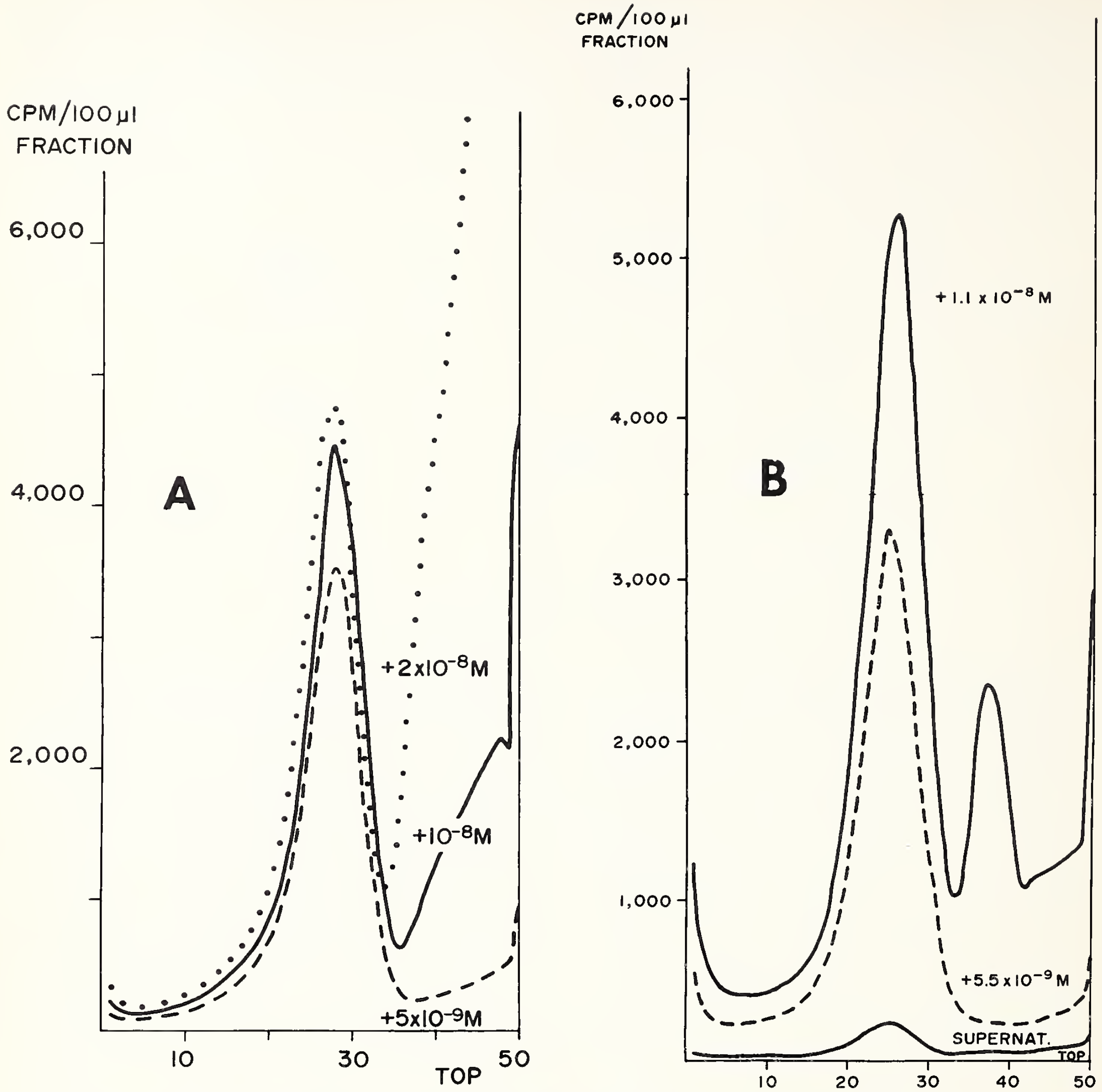


TEXT-FIGURE 2.—Interaction pathway of estradiol (E) in uterine cells.

BINDING OF ESTROGENS IN EXPERIMENTAL MAMMARY TUMORS

Although the uptake of estradiol by normal mammary gland is rather low, probably because of the large content of adipose tissue, careful investigations by both biochemical and autoradiographic techniques have demonstrated that specific estradiol binding, similar to that in the uterus and vagina, occurs in the mammary glands of the rat *(8, 9)*, the mouse *(10)*, and man *(11)*. More extensively studied have been experimental mammary tumors, such as those induced in the Sprague-Dawley rat by the feeding or injection of carcinogenic hydrocarbons *(12, 13)*. These hormone-dependent tumors resemble the uterus and vagina (text-fig. 4) in their affinity for estradiol (but not estrone), which is incorporated without chemical transformation, and in the sensitivity of this uptake to nafoxidine and similar binding inhibitors *(14–19)*. A small fraction (10–12%) of these induced tumors appear to be nondependent, since they do not regress after ovariectomy; the uptake of estradiol by these nonregressing tumors is considerably smaller than that of the dependent ones *(16)*. A detailed study *(20)* of the interaction of estradiol with 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary tumor has demonstrated that this interaction proceeds by a two-step mechanism in which an 8S receptor protein in the cytosol, dissociable into 4S subunits, is the precursor of the 5S estradiol-receptor complex extractable from the nucleus. One difference between tumor and uterus is that in tumor a greater proportion (85–90%) of the estradiol incorporated is found in the nuclear fraction.

On exposure to dilute solutions of tritiated estradiol *in vitro*, slices of hormone-dependent rat mammary tumor show a marked uptake of radioactive hormone which, similar to that of the uterus, is markedly reduced in the presence of binding inhibitors such as nafoxidine *(19–21)*. As illustrated in text-figure 5, those tumors which do not regress after ovariectomy can be readily distinguished from the "hormone-dependent" tumors both by the magnitude of the estradiol uptake and by the degree of sensitivity to the inhibitor. As with the uterus, adding tritiated estradiol to the cytosol fraction of a tumor homogenate gives rise to an estradiol-receptor complex which sediments as a well-defined 8S peak on sucrose density gradient ultracentrifugation and which, in the presence of saturating amounts of estradiol, can serve as a measure of the total 8S receptor content of the tumor cytosol *(20)*. In hormone-dependent tumors, where slices show a



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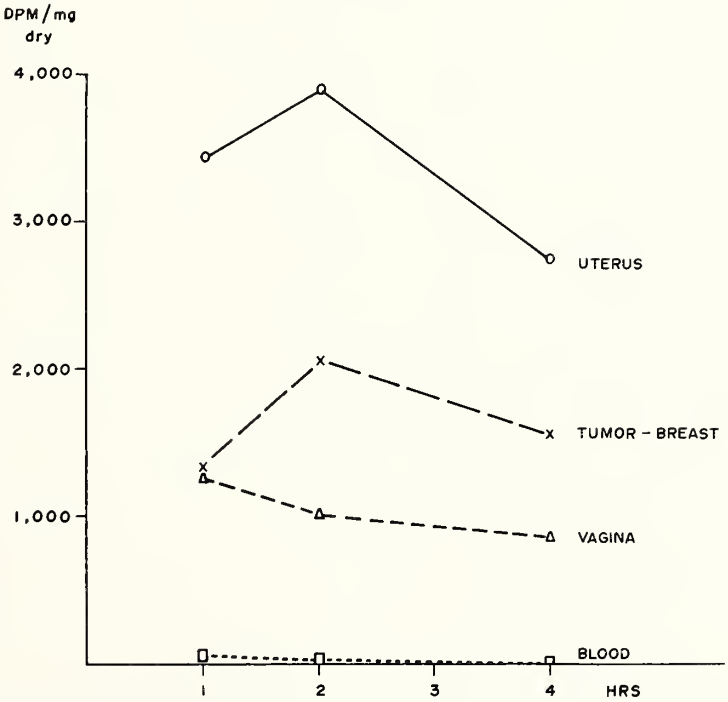
TEXT-FIGURE 3.—Saturation of the 8S receptor protein by addition of tritiated estradiol to rat uterine cytosol. A: Uteri from untreated rats were stirred in Krebs-Ringer-Henseleit buffer, *p*H 7.3, for 30 minutes to remove serum protein before they were homogenized in Tris-ethylenediaminetetraacetate. B: Uteri were excised 15 minutes after subcutaneous injection of 50 ng estradiol-6,7-^{3}H and homogenized directly. Centrifugation was for 7 hours at 216,000 × *g* in a 5 to 20% sucrose gradient.

large specific estradiol uptake, the 8S sedimentation peak is substantial, whereas those tumors which do not regress on ovariectomy show only small 8S receptor contents.

ESTROGEN RECEPTORS IN HUMAN BREAST CANCER

After the original demonstration of striking remission of advanced breast cancer following removal of the adrenal glands *(22)*, it has become recognized that hormone deprivation, accomplished by either adrenalectomy or hypophysectomy, can provide effective therapy for advanced breast cancer in postmenopausal patients. Unfortunately, however, clinical experience has shown that only a small fraction (20–25%) of the postmenopausal breast-cancer patients have tumors responding to endocrine ablation. A method of predicting *a priori* which cancers are of the responsive type would greatly enhance the usefulness of ablative therapy by restricting its application to those patients in which it has a reasonable chance of success and sparing most of the patients the trauma of a major surgical procedure which cannot help them and which renders them less able to tolerate alternative types of therapy.

Soon after the original observations of the striking affinity of estrogens for target tissues, Folca, Glascock, and Irvine *(23)* injected tritiated hexestrol into breast cancer patients, who were about to undergo adrenalectomy. They observed that the uptake of radioactive hormone by the tumor, as compared to that of skeletal muscle, was greater in 4 patients who experienced remission than in 6 patients who did not. These results, together with the accumulation of knowledge concerning the estrogen receptor proteins of normal target tissues and the greater receptor content of dependent rat mammary tumors, as compared to nondependent ones, suggested that an estimation of the receptor content of a breast cancer specimen might provide a valuable guide for predicting response of that patient to ablative therapy. Accordingly, an investigation was undertaken both of patients with metastatic disease, to correlate their response to adrenalectomy or hypophysectomy, and of mastectomy patients, to



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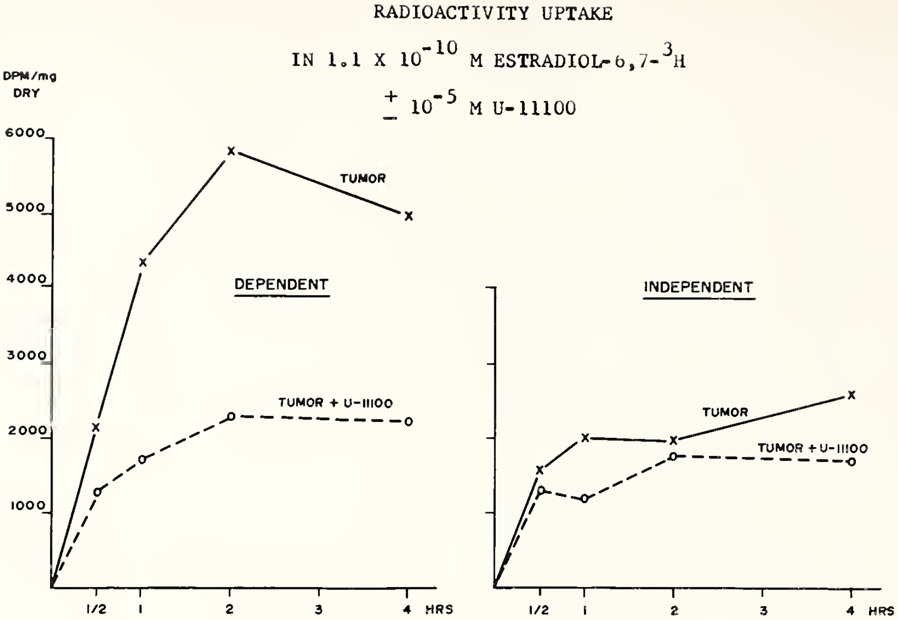
TEXT-FIGURE 4.—Concentration of radioactivity in dried tissues of tumor-bearing rats ovariectomized 2 days before intravenous injection of 120 ng (23 μCi) estradiol-6,7-^{3}H.



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Text-figure 5.—Comparison of hormone-dependent and hormone-independent rat mammary tumor slices in uptake of radioactivity on exposure to 0.1 nM estradiol-6,7-^{3}H in presence and absence of 10 μM nafoxidine (Upjohn 11,100). Each *point* is the median for 5 slices. Dependent tumors were taken from 42-week-old rats receiving DMBA (3 × 2 mg, iv) at age 50–56 days and ovariectomized 24 hours before the experiment; nondependent tumors were those continuing to grow in similar rats ovariectomized 10 weeks before the experiment.

characterize the primary tumor for future correlation with the response to endocrine therapy in those patients in which the cancer might recur.

Materials and Methods

Tumor specimens were obtained as soon as possible after excision (usually 15–30 minutes) and dissected free of necrotic, adipose, or other extraneous tissue with the advice of a pathologist.[7] For uptake studies, 0.5-mm slices of fresh tumor tissue were cut in a Stadie-Riggs apparatus and kept in cold Krebs-Ringer-Henseleit glucose buffer (KRH), *p*H 7.3, until all sections had been prepared. Half the slices, selected at random, were transferred to a beaker containing 300 ml of 0.1 nM estradiol-6,7-^{3}H (specific activity 57 Ci/mmole) in KRH buffer which had been equilibrated in a water bath at 37°C; the remaining tissue was placed in a similar solution which also contained an estrogen antagonist, either nafoxidine or Parke-Davis CI-628, in 10 μM concentration. The two solutions were stirred magnetically in the constant-temperature bath. At appropriate intervals, usually 30 minutes, 5 or more slices were removed from each solution and blotted, dried from the frozen state, and weighed. The content of tritiated hormone was determined by combustion (earlier by a modified Schöniger technique, more recently in a Packard Model-300 oxidizer) to yield tritiated water, which was counted in a liquid scintillation spectrometer.

For sedimentation studies, the weighed tumor specimens (except for a few which were homogenized while fresh) were frozen in liquid nitrogen, and the frozen tissue was shattered under liquid nitrogen in a Thermovac stainless-steel pulverizer. The resulting powder was thawed and carefully homogenized in 4 volumes of cold 10 mM Tris buffer, *p*H 7.5, containing 1.5 mM ethylenediaminetetraacetate (EDTA), using an all-glass homogenizer and 15-second homogenization periods, each followed by a 45-second cooling period. The resulting homogenate was centrifuged at 2°C for 30 minutes at 300,000 × *g* to yield the cytosol fraction. Portions (150 μl) of this cytosol were treated with 50 μl of Tris-EDTA buffer either with or without 1 μM Parke-Davis CI-628; after standing 10 minutes at 0°C, 50 μl of buffer containing tritiated estradiol was added. In more recent experiments, a 2.5 nM solution of estradiol was used, giving a final concentration of 0.5 nM; in earlier experiments somewhat higher estradiol concentrations were used. A 200-μl portion of each solution was layered carefully on a cold, preformed, linear sucrose gradient (5–20% or preferably 10–30%), also containing Tris-EDTA buffer, *p*H 7.5. After centrifugation for an appropriate period (usually 12–13 hours for 10–30% gradients or 7–8 hours for 5–20% gradients either at 300,000 × *g* in an International SB-405 rotor or at 308,000 × *g* in a Spinco SW-56 rotor), successive 100-μl fractions were drawn off by needle puncture of the bottom of the centrifuge tube, by use of a Hamilton repeating dispenser and paraffin oil to displace the fractions through the needle. The tritium content of each fraction was determined by direct liquid scintillation counting in a toluene-Triton X-100 scintillation medium.

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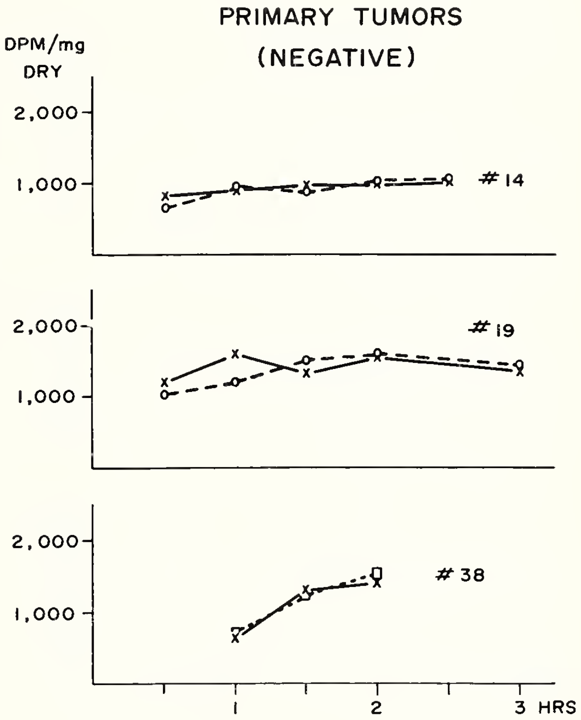
[7] We are grateful to Dr. T. Sugiyama and Dr. R. Fukunishi for their generous assistance in selecting the tissue to be studied.

For determination of estradiol binding with the Sephadex G-25 procedure *(24)*, portions of the tumor cytosols were diluted 1 : 10 with buffer containing various amounts of tritiated estradiol. Each was passed through a 20-ml column of Sephadex to remove the free hormone; the radioactivity in the void volume (as determined by addition of the colored macromolecule, blue dextran) was obtained for scintillation counting by ether extraction of each eluate.

Results

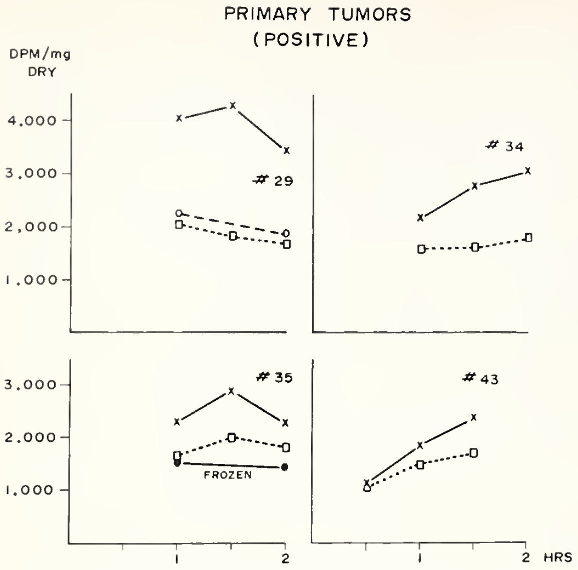
When slices of primary breast tumor were examined for estradiol affinity by the uptake-inhibition procedure, they were found to fall into two classes: 1) those showing a low uptake not depressed in the presence of nafoxidine or Parke-Davis CI-628 (negative pattern, text-fig. 6) and 2) those showing a substantially larger incorporation which was reduced by the inhibitor (positive pattern, text-fig. 7). In most instances a clear-cut distinction between positive and negative patterns could be made; a few cases showed borderline patterns. Metastatic tumors present similar negative (text-figs. 8, 9) and positive (text-fig. 10) patterns. For one patient (#24) with multiple skin metastases, individual tumors could be examined; all were negative (text-fig. 9). Usually, but not always, positive metastatic tumors showed a higher total level of estradiol incorporation than primary tumors, possibly because the primary tumors usually contained adipose tissue which has little affinity for the hormone.



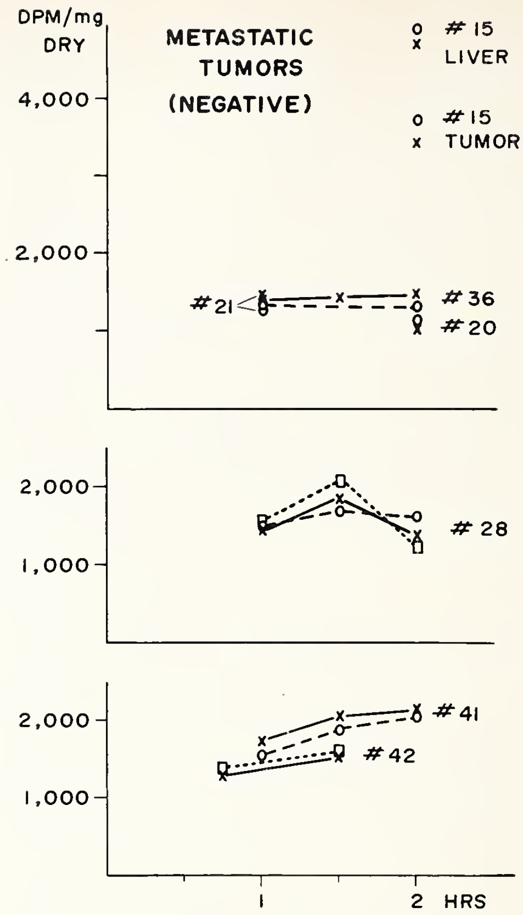
Text-figure 6.—Negative radioactivity uptake patterns in slices of human primary breast cancers in incubation with 0.1 nM estradiol-6,7-^{3}H as described in the text. In this and subsequent text-figures of this type, x——x indicates uptake in estradiol alone; O — — — O, in the presence of 10 μM nafoxidine; and □ — — — □, in the presence of 10 μM Parke-Davis CI-628. Each *point* is the median value for at least 4 slices, usually 5 or more.

In earlier experiments, incubation periods of up to 4 hours were used, but tumor slices often lost their binding capacity after longer incubation periods, so that the 4-hour time point (text-fig. 5) and, in some cases, even the 2-hour time point (text-fig. 6) was lower than the one preceding it. In more recent experiments, incubations were usually carried out for a maximum of 90 minutes and in no case for more than 2 hours.

Thus, the uptake of estradiol by tumor slices permits a convenient characterization of human breast cancers, primary or metastatic, as those which resemble the uterus in possessing significant estro-



TEXT-FIGURE 7.—Positive uptake patterns in slices of human primary breast cancers. In one instance a portion of the specimen had been frozen and thawed before slicing.

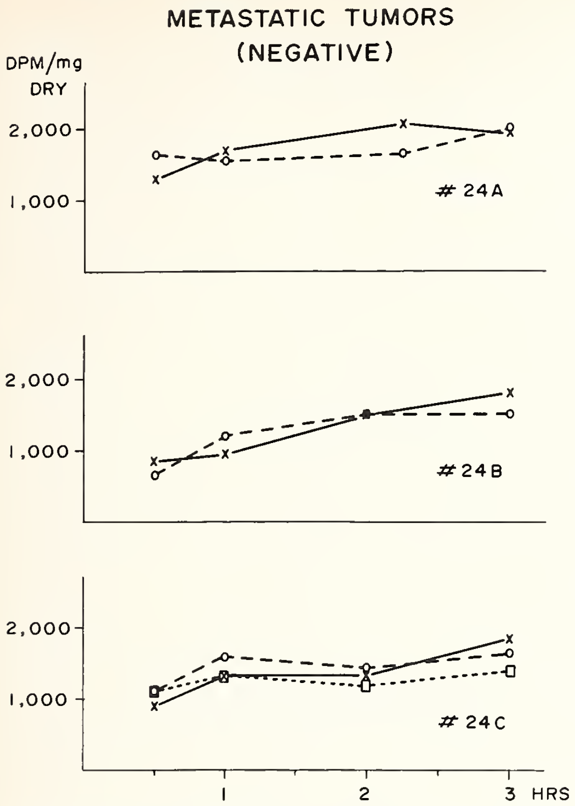


TEXT-FIGURE 8.—Negative uptake patterns in slices of metastatic breast cancers. Metastases: #20, 28, 36, 42, skin; #21, adrenal; #41, lymph node. Patients 20 and 21 had only enough tumor specimen for one time point.

gen affinity and those which do not. But this procedure suffers from two limitations. First, the tumor specimen must be sufficiently large (weighing >0.5 g) to permit the preparation of at least 20, and preferably 30, 0.5-mm slices, so that differences due to heterogeneity of the tumor can be averaged out. Although sample size usually is not a problem with primary tumors, many metastatic cancer specimens are not large enough to permit definitive evaluation by this procedure. Moreover, the determination cannot be carried out on frozen tissue. After freezing, even in liquid nitrogen, the positive tumor slices, as well as uterine tissue, show markedly reduced estradiol uptake, in many instances to the level of nonspecific binding (text-figs. 7, 10). Freezing of the tissue does not destroy the receptor proteins but seems to render the cells permeable to the 8S cytosol receptor, which leaks out into the medium during incubation and is not available for uptake of estradiol and fixation in the nucleus.

A method of tumor characterization, applicable to small (200 mg) samples of either fresh or frozen tumor, is provided by the direct estimation of the cytosol receptor content by sucrose density gradient centrifugation. As previously described for the uterus (text-fig. 3), addition of saturating amounts of estradiol to the cytosol fraction of a tumor homogenate gives rise, in a tumor with a positive uptake pattern, to an 8S complex which can be estimated by the magnitude of the 8S sedimentation peak. As illustrated in text-figure 11, the positive metastatic tumor (#32) showed a well-defined 8S peak when either fresh or frozen tissue was examined, in contrast to the negative tumor (#24B) which showed no radioactivity in the 8S region.

As previously described for the uterus (text-fig. 3B), if serum proteins are present, as is sometimes the case in human breast cancers, nonspecific binding will be observed in the 4.5S region after the specific receptor has been saturated. This peak is clearly distinguishable for the specific 8S peak, both by its position on the gradient and by its



Text-figure 9.—Negative uptake patterns in slices of 3 different skin metastases taken from the same patient.

insensitivity to Parke-Davis CI-628, as described below. When present, this nonspecific binding is usually in such amounts that it is not saturated by concentrations of estradiol far in excess of those required to saturate even the highest levels of 8S receptor encountered in tumor tissue (text-fig. 12).

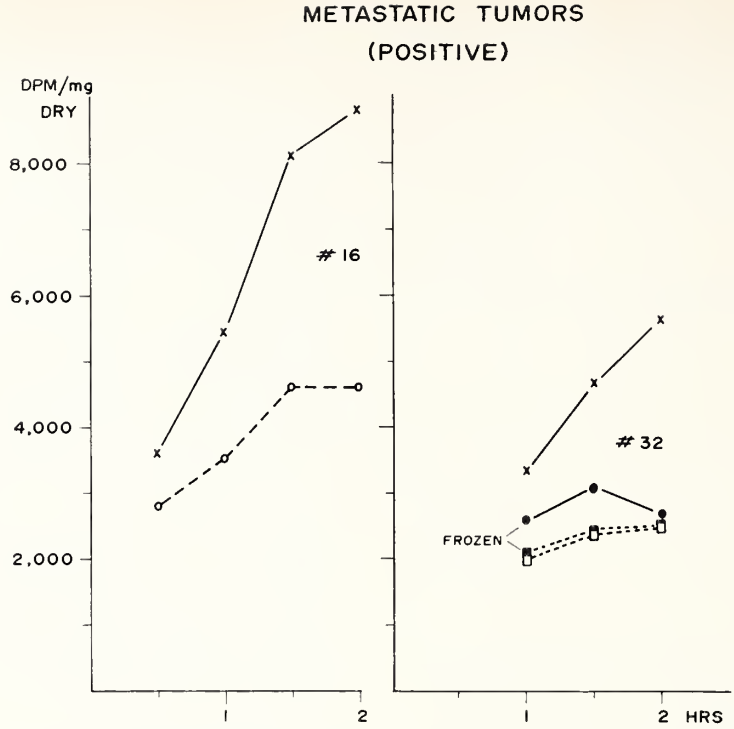
In our earlier sedimentation experiments, sucrose gradients of 5–20% and estradiol concentrations of 1–2 nM were used. Under these conditions, it was found that the 8S complex from human breast cancers, unlike that of uterus, sometimes tended to decompose partially on the gradient. For example, patient 51 (text-fig. 13) showed a poorly defined peak, which still permitted distinction from the negative pattern of patient 56, in agreement with the comparative uptake patterns of these 2 patients (text-fig. 14). Later it was found that this tendency to decompose during centrifugation was much less when a 10–30% sucrose gradient was used. With some tumors, the 8S complex tended to aggregate partially; as illustrated in text-figure 15, the characterization of patient 66 as having a positive sedimentation pattern was sharpened considerably by carrying out the study both in the presence and absence of Parke-Davis CI-628, which eliminated the specific binding in both the 8S peak and the aggregate and resulted in a pattern very much like the negative tumor, #67.

From the experience gained as the investigations progressed, a more satisfactory set of experimental conditions for density gradient studies of tumors was adopted. These included the use of 10–30% sucrose gradients, which gave sharper 8S peaks less subject to decomposition, the use of 0.5 nM rather than 1 or 2 nM estradiol, so that there was less excess radioactivity at the top of the tube, and the comparison of the sedimentation pattern obtained with and without the inhibitor, Parke-Davis CI-628, which eliminates specific binding but has no effect on nonspecific binding to serum proteins.

Some of the various types of positive sedimentation patterns obtained with primary and metastatic breast cancers are illustrated in text-figures 16 and 17 with uptake patterns for the same patients shown in text-figures 14 and 18. In addition to the 8S peak, some human breast cancers (#68, #86, #89) showed a second peak of specific binding, sensitive to the inhibitor, which appeared in the 4S region. This may represent the 4S subunit of the 8S receptor, which has been shown for the adult rat uterus *(25)*, as well as for the adult human uterus *(26, 27)*, to differ from the immature rat uterus, in that part of the receptor exists in the 4S form, even at low-ionic strength. In any case, specific binding in either the 8S or 4S region can be distinguished from the nonspecific 4.5S binding by its sensitivity to the Parke-Davis inhibitor.

The lymph node from patient #90 represented a tumor containing an unusually high concentration of cytosol receptor, as indicated by its almost complete utilization of the 0.5 nM estradiol. Apparently, because of the large amount of complex formed, the usual amount of inhibitor employed did not completely eliminate the 8S peak. The primary tumor from this patient also showed a large sedimentation peak as well as substantial uptake of estradiol by tumor slices (text-fig. 18).

In seeking a simpler procedure for evaluating small amounts of frozen tumor specimen, which does not require the use of an ultracentrifuge, many of the tumor cytosols were also studied by the method of Puca and Bresciani *(24)*, in which por-



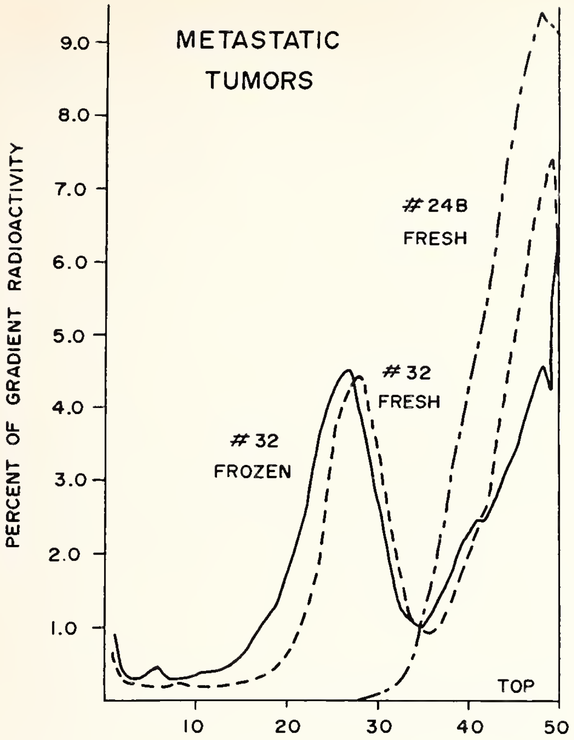
Text-figure 10.—Positive uptake patterns in slices of adrenal metastases. For #32, a 0.05 rather than 0.1 nm solution of estradiol-6,7-^{3}H was used for the incubation.

tions of diluted cytosol were treated with different amounts of tritiated estradiol and, after equilibration, the excess free estradiol was removed by filtration through Sephadex G-25. When the bound radioactivity was plotted against the total added (text-fig. 19), a negative tumor (#67) gave a curve which extrapolated essentially to the origin, in contrast to a tumor containing receptor (#66) which showed a biphasic curve consisting of a strong, saturable specific binding superimposed on a weaker, nonspecific binding, which continued to increase as more hormone was added. Although this technique provides a simple means for estimating estradiol binding and for distinguishing a specific from a nonspecific interaction, on a routine basis it did not prove as reliable as the sucrose gradient sedimentation procedure.

Clinical Correlations

As seen in table 1, the 51 mastectomy patients studied divided equally between positive and negative patterns, with two borderline cases. Disease subsequently recurred in 5 of these patients, one of whom was subjected to adrenalectomy and failed to respond. This patient's primary tumor had shown a negative pattern in the laboratory test. If tumor recurs in others of this group, especially those with positive patterns, hopefully some of these patients will undergo endocrine treatment so that their responses can be correlated with their tumor patterns.

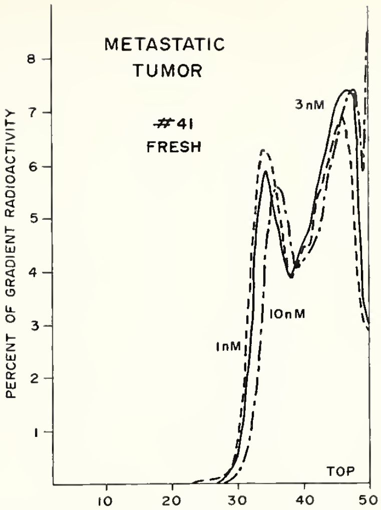
Studies were done on 33 patients undergoing endocrine therapy for advanced breast cancer (tables 1, 2). Of these patients, 28 had adrenalectomy (7 with accompanying oophorectomy and 1 with subsequent hypophysectomy), 3 had hypophysectomy, 1 had radiation castration, and 1 received depo-testosterone. Negative patterns were seen in 19 patients, of which 17 failed to respond; 1 patient died in the immediate postoperative course and 1 experienced remission. Of 11 patients showing positive patterns, 5 experienced significant remissions, 2 failed to respond, and 4 died in the immediate postoperative period from overwhelming carcinomatosis. Of 3 patients with borderline patterns, 1 died in the postoperative period and 2 did not show objective remission, although 1 of these did show a subjective remission, living for 18



Text-figure 11.—Sedimentation patterns of cytosol fractions of homogenates of metastatic breast cancers described in text-figures 9 and 10. Estradiol concentrations: #24B fresh, 5 nM; #32 fresh, 4 nM; #32 frozen, 3 nM. Centrifugation: #24B, 8 hours at 186,000 × *g*; #32, 7 hours at 216,000 × *g* (in different runs), in 5–20% sucrose. All gradients also contained 10 mM Tris buffer, *p*H 7.5, with 1.5 mM ethylenediaminetetraacetate.

months despite evidence of progressing disease.

From the experience so far, patients whose breast cancers lack the estrogen receptor substance have very little chance of responding to endocrine therapy and probably can be spared the trauma of ablative surgery. Of those patients who possess estrogen-binding proteins, some, but not all, will benefit from such treatment. The failure of some patients with positive patterns to respond is understandable, if one considers that it is possible to have a mixed population of positive and negative metastases. In such cases, the pattern observed may be either positive or negative, depending on which metastasis is obtained for study, but the patient will be a failure because the negative metastases will be unresponsive.



Text-figure 12.—Sedimentation pattern of cytosol from negative metastatic tumor described in text-figure 8 in presence of 1, 3, and 10 nM estradiol-6,7-^{3}H. Centrifugation: 7 hours at 258,000 × *g* in 5–20% sucrose. Under these conditions, the 8S peak would be expected about fraction 20.

The existence of some "false positives" does not represent a serious problem, because, without any selective criterion, these patients would undergo treatment. It is important that there be no significant number of "false negatives," because it is on the basis of a negative test that a decision would be made not to carry out treatment. So far, the correlation between lack of receptors and lack of response has been good, and one can hope that this relation will continue to hold, as a substantial number of cases are accumulated.

Addendum

Since the original presentation of this report, additional patients have been included in the study. Of a total of 84 primary tumors investigated, 39 showed the presence of estrogen receptor, 38 were negative, and 7 were borderline. The median age of patients with positive primary tumors was 64 (range 34–83) and with negative tumors 52 (range 32–86). Of 40 metastatic tumors examined, 12 were positive, 25 negative, 2 borderline, and one patient with multiple metastases showed both positive and negative specimens.

Of 58 patients with metastatic disease studied, 42 received some type of endocrine therapy under conditions permitting evaluation of clinical response. In 14 patients the primary tumor was still present and used for characterization; in 28 others, metastases were examined. Of 29 patients showing no

Table 1.—Summary of all patients studied

51—Mastectomy, *, †, pattern ‡	33—Endocrine therapy	
	Pattern ‡	Response
25, —	19, —	17 Failure
		1 Remission
		1 Surgical casualty§
24, +	11, +	2 Failure
		5 Remission
		4 Surgical casualty
2, ±	3, ±	2 Failure
		1 Surgical casualty

*In addition to these 51 primary malignancies, 1 benign breast tumor showed a negative pattern as did 1 cancer of the male breast.

† One mastectomy patient showing a positive pattern underwent prophylactic adrenalectomy 6 weeks later.

‡ —indicates negative pattern; +, positive pattern; and ±, borderline pattern.

§ Surgical casualty signifies that the patient, usually debilitated and in carcinomatosis, did not survive the immediate postoperative period so that the response could not be evaluated.

Table 2.—Type of endocrine therapy

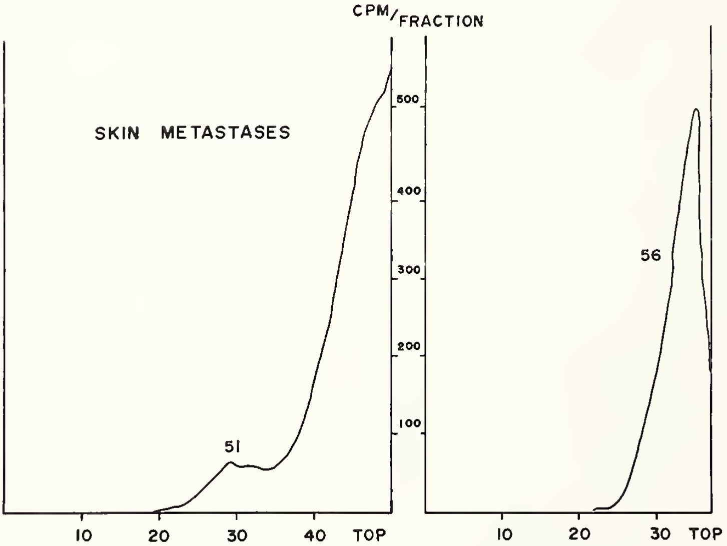
Endocrine therapy	Remission	Failure	Surgical casualty §
28—Adrenalectomy *, †, ‡			
+	4	2	4
—	1	13	1
±		2	1
3—Hypophysectomy			
+	1		
—		2	
1—Radiation castration			
—		1	
1—Testosterone			
—		1	

* Of these patients undergoing adrenalectomy, 5 with negative patterns and 2 with positive patterns had accompanying (or in one case prior) oophorectomy while another with negative pattern had radiation castration.

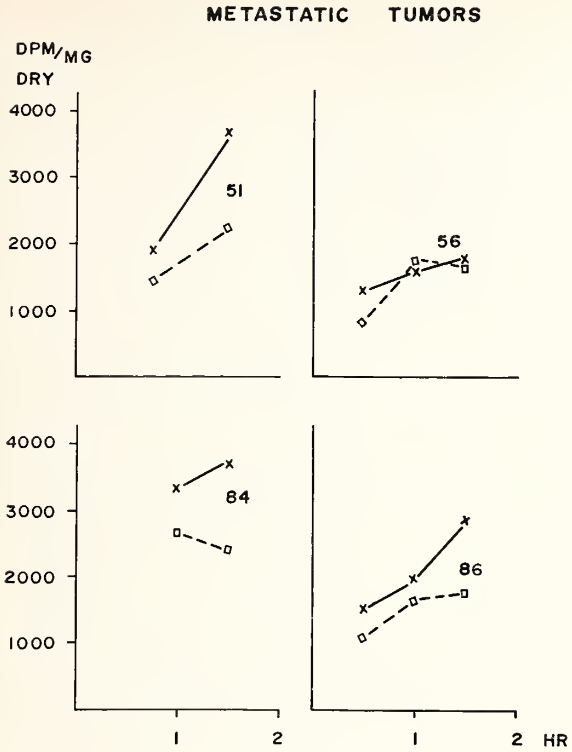
† In 6 of these adrenalectomy patients, the primary tumor was still present and was used for the study. Of these, 2 positive patterns were associated with remissions, whereas 3 negative patterns and 1 borderline pattern were associated with failure.

‡ + indicates positive pattern; —, negative pattern; and ±, borderline pattern.

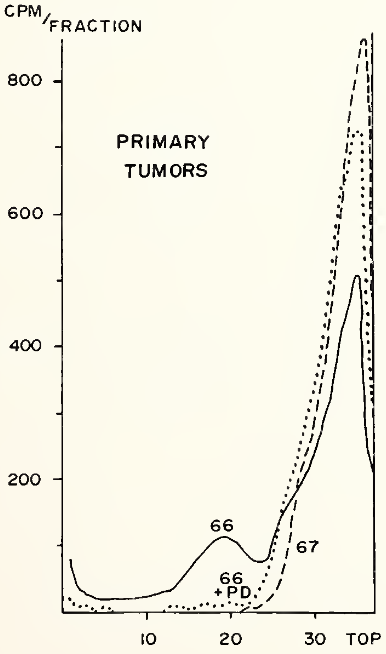
§ Surgical casualty signifies that the patient, usually debilitated and in carcinomatosis, did not survive the immediate postoperative period so that the response could not be evaluated.



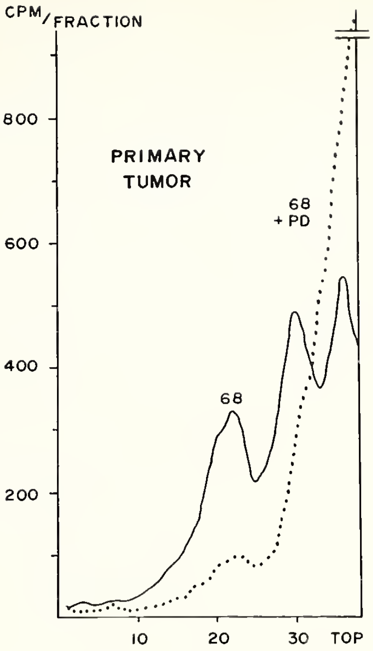
Text-figure 13.—Sedimentation patterns of cytosol from two specimens of skin metastases, described in text-figure 14, by use of 1 nM estradiol-6,7-^{3}H for #51 and 0.5 nM for #56. Centrifugation: #51, 9 hours at 165,000 × g; #56, 9 hours at 300,000 × g, in 5–20% sucrose.



Text-figure 14.—Three positive uptake patterns and one negative uptake pattern for slices of metastatic tumors. Metastases: #51 and 56, skin; #84, liver; and #86, chest wall.

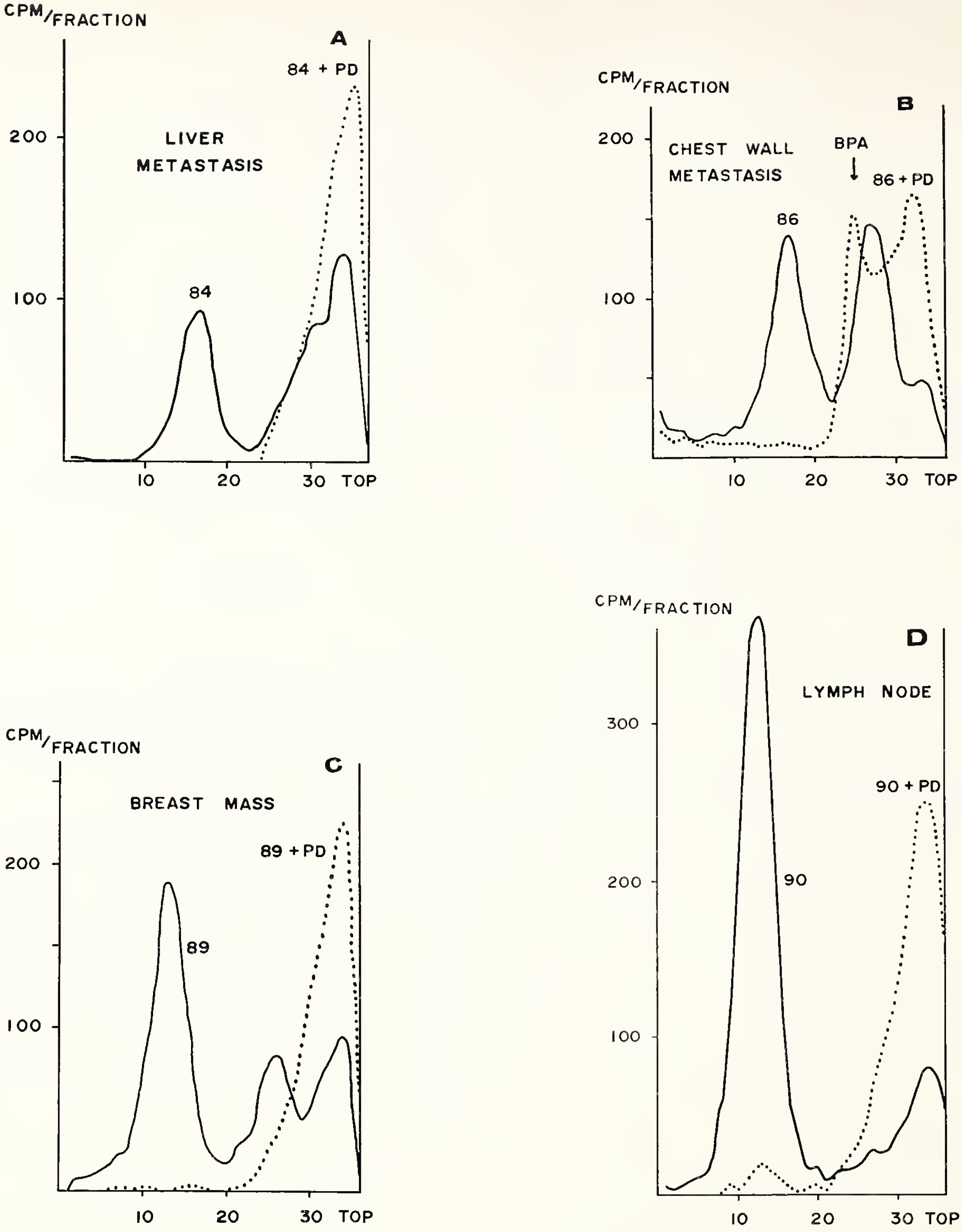


Text-figure 15.—Sedimentation patterns on cytosol from primary tumors by use of 1 nM estradiol-6,7-^{3}H with and without 2 μM Parke-Davis (PD) CI-628. Centrifugation: #66, 7.5 hours at 300,000 × g; #67, 10 hours at 200,000 × g, in 5–20% sucrose. The curve for #66 + PD was practically identical with that shown for #67 alone.

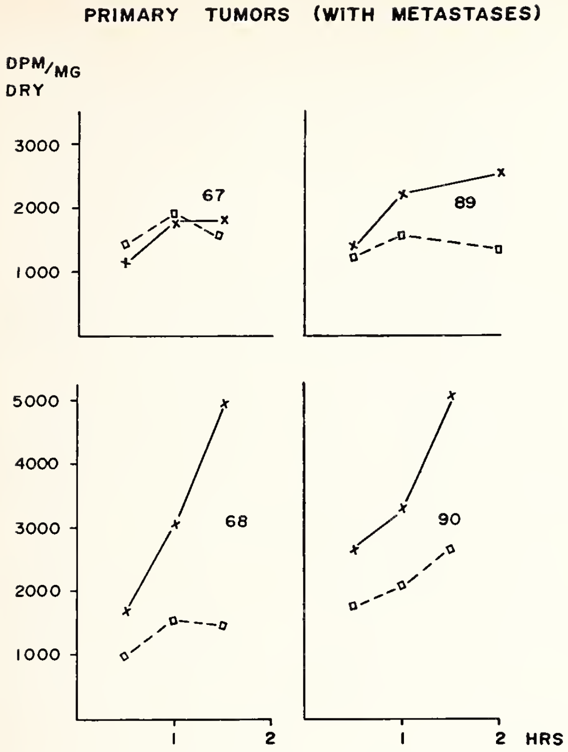


Text-figure 16.—Sedimentation pattern of cytosol from primary tumor, described in text-figure 18, by use of 2 nM estradiol-6,7-^{3}H with and without 0.2 μM Parke-Davis (PD) CI-628. Centrifugation: 7.5 hours at 308,000 × g, in 5–20% sucrose.

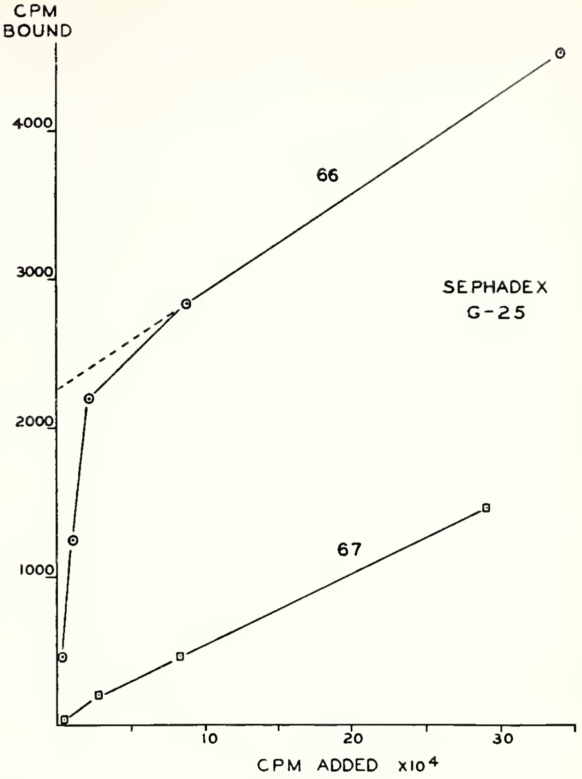
distinct evidence of estrogen receptor (26 negative, 3 borderline) only one patient experienced remission after therapy (adrenalectomy). In contrast, 10 of 13 patients whose cancers contained estrogen receptor showed objective remission to endocrine therapy. These additional findings support the previous conclusion that breast cancer patients whose tumors lack the estrogen receptor probably can be spared the trauma of ablative endocrine therapy.



Text-figure 17.—Sedimentation patterns of cytosols from positive primary and metastatic tumors by use of 0.5 nM estradiol-6,7-^{3}H and 0.2 μM Parke-Davis (PD) CI-628. Centrifugation: #84, 12 hours at 308,000 × g; #86 and #90, 12 hours at 300,000 × g; #89, 13 hours at 300,000 × g, in 10–30% sucrose. In #86, BPA indicates position of bovine plasma albumin marker.



Text-figure 18.—Three positive uptake patterns and one negative uptake pattern for slices of primary tumors from patients who also had metastases.



Text-figure 19.—Estradiol-binding curves of diluted cytosols from two primary breast cancers: #66 shows a combination of specific and nonspecific association; #67 shows only nonspecific binding.

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Estrogens in Breast Tumors[1, 2]

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THE MODE of action of estrogens and androgens on malignant breast tumors has not been elucidated. While an indirect effect, such as the formation of growth activators or inhibitors elsewhere in the body, cannot be excluded, the possibility of a direct action on, and an associated concentration mechanism in, the tumor seemed worthy of study.

When this investigation was begun, available chemical methods for the determination of steroid hormones were insufficiently sensitive for the minute amounts present in small tissue samples. A technique involving constant intravenous infusions of radioactive tracer steroids of high specific activity was considered the most suitable for comparison of plasma and tissue steroid concentrations in individual patients *(1)*.

METHODS

The infusions were administered as constantly monitored drips of solutions of tritium-labeled steroid in saline containing human serum albumin to prevent excessive losses of tracer by adsorption. The infusion rates ranged from 3.3–14.3 μc/hour, and the total amounts of steroid administered were equivalent to <35 μg/24 hours. Infusions were continued for 3–6 hours just before and during surgery until the tissue samples were removed. Blood was collected at various times during the infusions and immediately after removal of the tissues. The amounts of tritium in the free steroid moiety of tissues and plasma were determined by extractions either with ether/ethanol (3:1), followed by partition between ether and water *(2)*, or with methylene chloride. Plasma samples could also be extracted directly with 30 volumes of a toluene scintillator. Chemical identification of estradiol was by thin-layer chromatography (TLC), oxidation to estrone, and further TLC *(3)*. Because of the small amounts of tritium associated with estrone from tissue extracts, its tentative identification was based on one TLC only. Tissue DNA was extracted by the method of Schneider *(4)*, and the ribose determined by the color produced with diphenylamine. Hydroxyproline, used as a measure of tissue collagen, was obtained by hydrolyzing the tissue overnight with 6 N HCl in sealed tubes and applying the color reaction described by Woessner *(5)*.

RESULTS

While testosterone or its metabolites did not appear to be concentrated in breast cancer tissue *(2)*, estradiol or its metabolites, or both, reached high concentrations relative to plasma in some, but not all, malignant breast tumors. This is illustrated in text-figure 1, which also shows the range found for adipose tissue (mostly collected from the breast) and results for a number of malignancies at other sites.

Text-figure 2 shows tissue/plasma ratios of free steroid radioactivity for normal tissues, together with the range for breast cancer. Only liver appeared to concentrate estradiol or metabolically related free steroids, but this finding requires confirmation. The low results for normal breast tissue (collected from premenopausal women) raise the question whether "target tissues" necessarily concentrate the active hormone. The category "other tissues" includes skin, bladder, adrenal cortex, adrenal medulla, ovary, stomach, intestine, and pancreas. Of the benign breast lesions studied, an intraduct papilloma was found to have a high tissue/plasma ratio (6.7) of radioactivity after infusion with estradiol. Mastitis, a fibroadenoma, and a lipoma from a breast had low ratios. Benign growths at other sites (intestinal polyp, gastric ulcer, a lump from an ear) did not concentrate estrogens.

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[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

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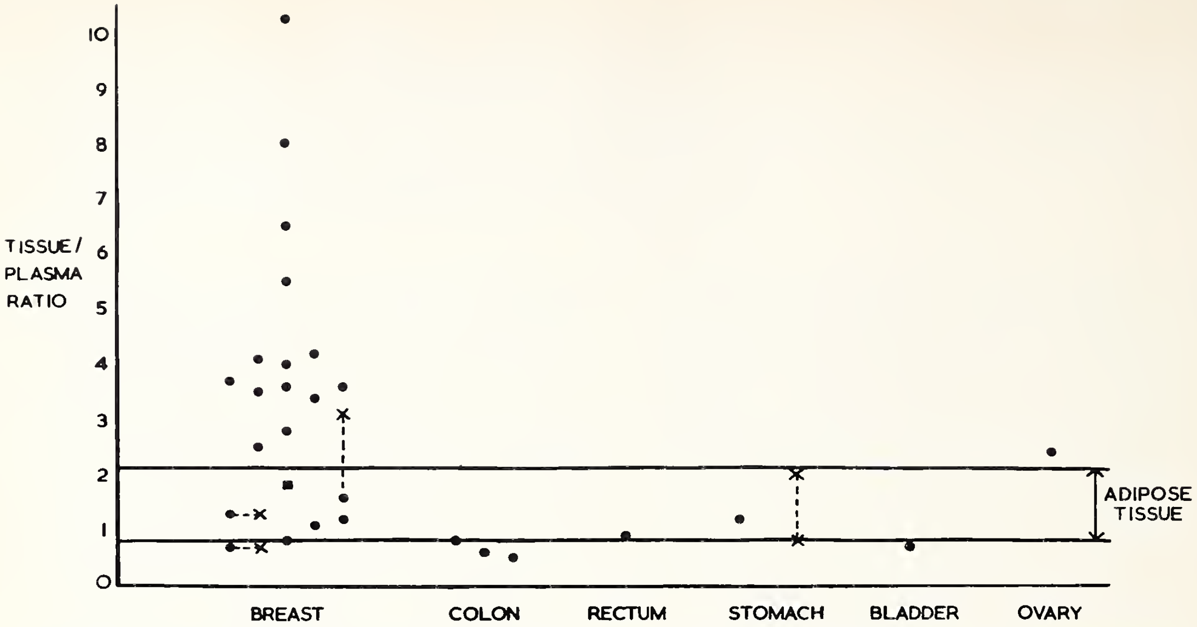
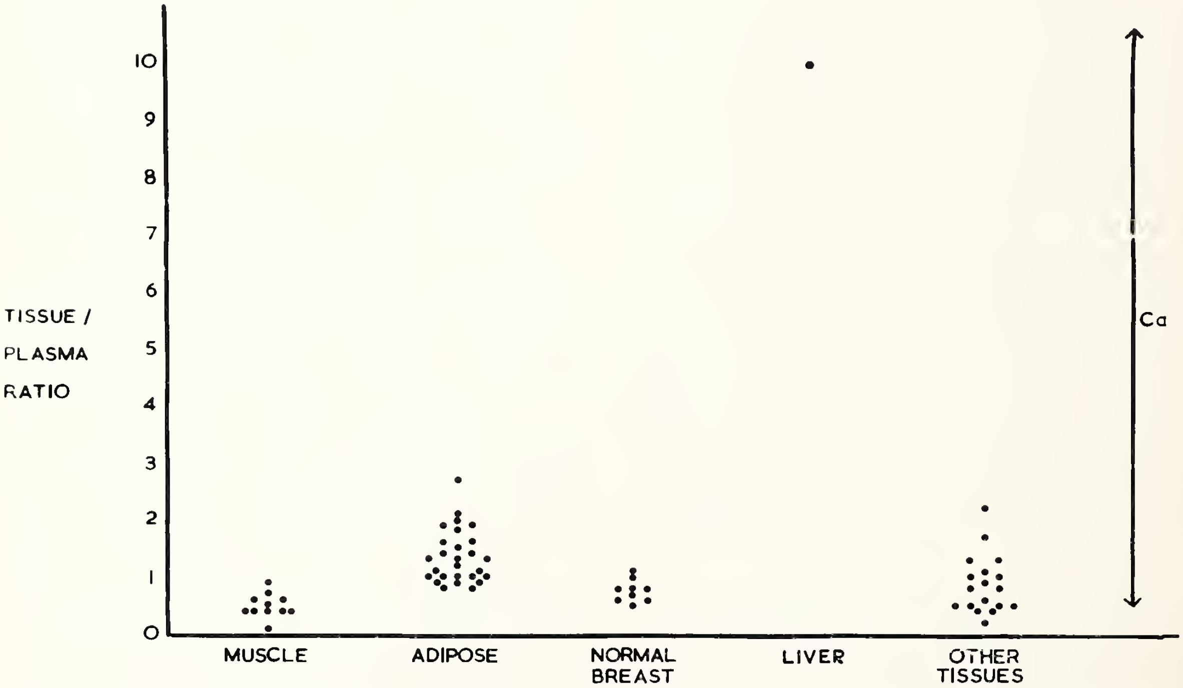


TEXT-FIGURE 1.—Tissue/plasma ratios of free steroid radioactivity for malignant tumors after infusion of tritium-labeled estradiol. ●: Primary growth; x: metastases (connected by *broken lines* to *points* for the primary tumor or another metastasis in the same patient); and ■: a colloid carcinoma of the breast.

Since the ability of breast cancer tissue to concentrate estradiol or its metabolic products, or both, appeared to be both tissue- and steroid-specific, the chemical nature of the radioactive component in the tumor was examined. In tumors with both high and low estrogen concentrations, most of the



TEXT-FIGURE 2.—Tissue/plasma ratios of free steroid radioactivity for normal tissues after infusion of tritium-labeled estradiol. The range for breast cancer tissue is shown on *right*.

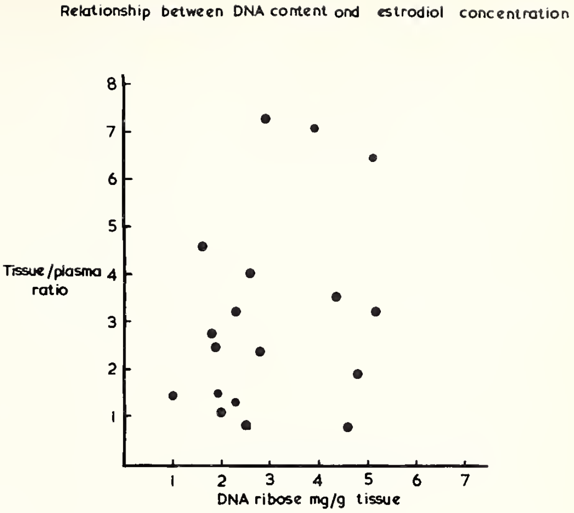
free steroid radioactivity was accounted for by estrone and estradiol, with one exception (patient 1, table 1). An appreciable proportion of estrone was found in 2 of 9 tumors examined (patients 2 and 4, table 1). Since both tumors contained little estrogen, its absolute concentration was low. Thus the high relative concentrations of radioactivity found were largely associated with estradiol. However, it is important to remember that, at the removal of the tissue, endogenous estrone present may not have approached equilibrium with radioactive estrone derived from infused estradiol.

Table 1.—Proportion of extractable free steroid radioactivity present as estradiol and estrone in tumor tissue

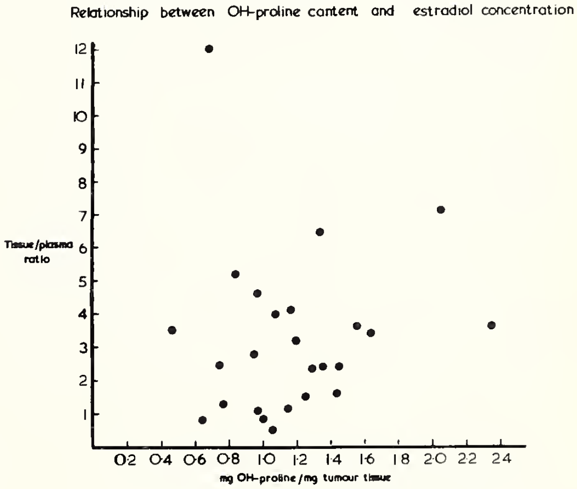
Patient	Tissue/plasma ratio of free steroid radioactivity	Estradiol (%)	Estrone (%)	Duration of infusion (minutes)
1	0.7	53.8	6.9	240
2	0.8	65.0	37.8	240
3	1.1	76.1	—	210
4	1.2	45.5	50.2	315
5	1.3	103.5	6.1	270
6	2.8	97.6	3.1	390
7	3.6	88.6	3.9	240
8	4.1	86.0	3.4	290
9	6.5	95.8	3.0	300
10	13.8	64.2	8.1	240

Possibly, the concentration of estradiol in cancer tissue was related only to the composition of the tumor. If it were dependent on the number of cells present or the proportion of connective tissue, then the relative estrogen concentration (expressed as the tumor/plasma ratio) would be correlated with DNA or hydroxyproline content. With small replicate samples of tumor tissue used, the coefficients of variation were 6.7% for DNA and 19.8% for hydroxyproline. The variability was partly due to heterogeneity of the tissue, and two or more replicate estimations were carried out on each tumor. The results are shown in text-figures 3 and 4. There was no significant correlation between tissue/plasma ratio of radioactivity and either DNA or hydroxyproline.

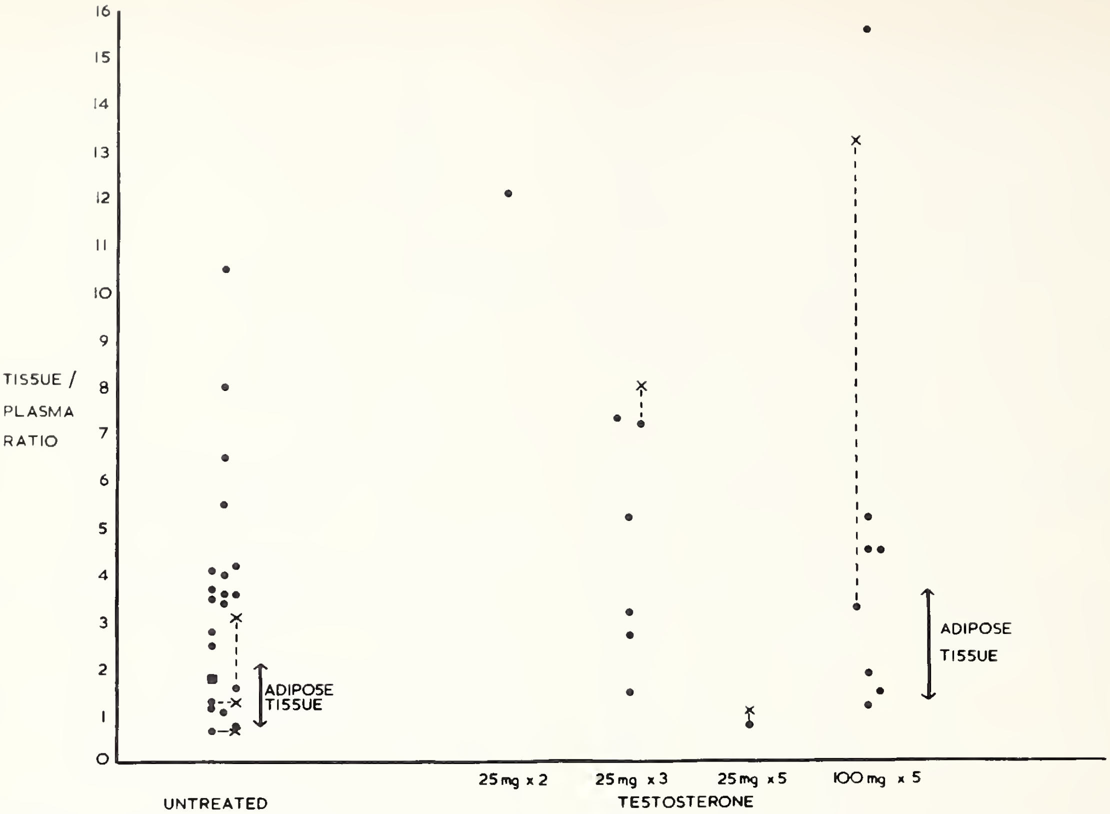
Whether estrogen concentration is related to the growth rate or hormone dependence of tumors is not known. A careful follow-up of patients whose estrogen concentration has been examined is in progress. Another approach has been made by estimating estrogen concentration after treatment with androgen. The results are shown in text-figures 5 and 6. Even after 5 daily injections of 100 mg testosterone propionate, several tumors produced high tissue/plasma ratios, and the only significant effect of this treatment was that the relative concentration of estrogen was increased in adipose tissue. After one injection of 100 mg of dromostanolone 18 hours before surgery *(6)*, the mean tumor/plasma ratio was 2.39 ± 1.57 (SD), range 0.50–4.58, N = 8, as compared to the mean for untreated patients of 3.54 ± 2.49 (SD), range



TEXT-FIGURE 3.—Relationship between DNA ribose content and tissue/plasma ratios of free steroid radioactivity of breast tumor tissue after infusion of tritium-labeled estradiol.



TEXT-FIGURE 4.—Relationship between hydroxproline content and tissue/plasma ratios of free steroid radioactivity of breast tumor tissue after infusion of tritium-labeled estradiol.



Text-figure 5.—Tissue/plasma ratios of free steroid radioactivity of breast tumor tissue from testosterone-treated patients after infusion of tritium-labeled estradiol.

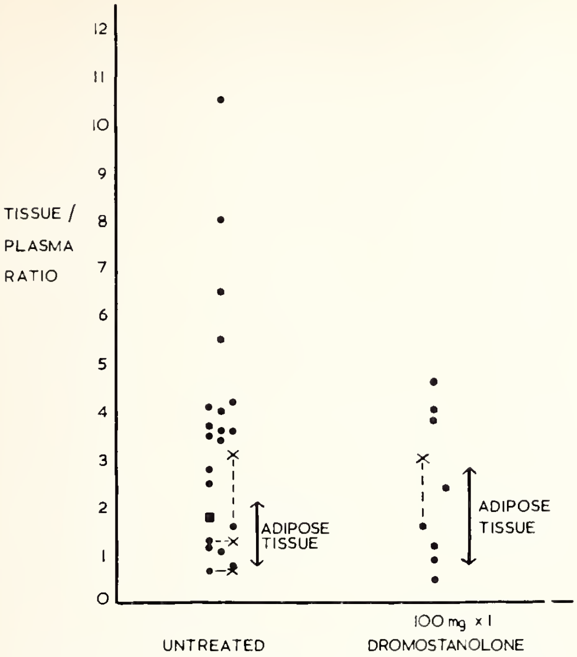
0.67–10.5, N = 21, and the difference was not significant. As in the untreated patients, estradiol was quantitatively the most important radioactive component (table 2).

DISCUSSION

That breast tumors may differ in steroid concentration is of some interest in relation to the etiology and progression of the disease. Until we have an extensive follow-up of patients, we cannot assess whether a high estradiol concentration can be used as a parameter in predicting response to treatment. Dao *(7)* reported differences in tumor content of steroid sulfating

Table 2.—Proportion of extractable free steroid radioactivity present as estradiol and estrone in tumor tissue. Patients treated with androgens

Treatment	Patient	Tissue/plasma ratio of free steroid radioactivity	Estradiol (%)	Estrone (%)	Duration of infusion (minutes)
Testosterone, 3 × 25 mg	11	2.7	90.0	10.5	290
	12	7.3	100.0	0	320
	13	7.9	91.5	5.2	255
Testosterone, 5 × 25 mg	14	0.8	75.6	24.9	245
	15	1.1	96.6	7.9	285
	16	1.3	65.6	34.1	270
	17	1.9	77.6	18.7	300
	18	4.5	84.4	18.7	240
Dromostanolone, 1 × 100 mg	19	2.5	90.0	21.4	300
	20	4.1	95.0	12.2	255
	21	4.6	78.9	2.4	270



Text-figure 6.—Tissue/plasma ratios of free steroid radioactivity of breast tumor tissue from dromostanolone-treated patients after infusion of tritium-labeled estradiol.

enzymes which may be important in defining suitable treatment. The two properties may or may not be related to each other.

The lack of correlation between the number of cells estimated by DNA content and estrogen uptake confirms an earlier finding *(2)* which involved careful microscopic examination of replicate tumor samples *(8)*. If the steroid is located outside the tumor cells, collagen does not appear to be involved in its concentrating mechanism. If it is contained within the cells, the question arises whether the cell population is homogeneous with respect to ability to concentrate the hormone, or whether different cell types exist in the same tumor—an interesting possibility that has not been investigated.

The small number of patients studied after short-term androgen treatment has not provided clear evidence that estradiol is displaced from the tumor. More prolonged treatment may or may not have such an effect. Ellis *et al. (6)*, using single injections of tritium-labeled estradiol, found a marked decrease in radioactivity content of tumors in patients treated with dromostanolone as compared with untreated patients. Apart from changes in tumor estrogen concentration, their method could reveal differences in estrogen metabolism and turnover after treatment *(1)*.

The finding that some patients have malignant breast tumors containing relatively large amounts of estradiol may be important in relation to response to endocrine treatment. Further studies on the location of the concentrating mechanism within the tissue and on the relation between tumor growth and estrogen concentration may throw some light on the mode of action of steroid hormones.

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Competition Between Steroids for Hormonal Receptor[1, 2, 3]

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WITH the advent of tritiated steroids of high specific activity, localized estrogen concentrations *in vivo* and *in vitro* have been described in the uterus, vagina, breast, and adenohypophysis after tissues from these sites were exposed to physiologic concentrations of free compounds. Jensen, Korenman, Gorsky, and others isolated cellular protein fractions from the cytoplasm and nucleus of uterine cells, which appear to account for the major part of local estrogen concentration, through avid binding to proteins, which have been termed "estrogen receptors." In the work of Mobbs, Terenius, King, Folca, Deshpande, and Bulbrook, the binding of tritiated hexestrol and estradiol-17β was carefully investigated in human and rat mammary neoplasms; a similar observation was made of predominant nuclear binding of estradiol. In induced mammary tumors of rats, those regressing after castration (hormone dependent) were reported to be more active in estrogen binding than those resistant to castration (hormone independent) *(1, 2)*.

Presence of 2-, 6-, and 16-Oxygenated Estrogen Metabolites in Body Fluid

In vivo, plasma and extracellular fluid bathing mammary tissues contain not only estradiol-17β (the most actively uterotropic ovarian estrogen) but also a variety of weakly estrogenic metabolites, which are far less uterotropic, having been oxidized at C2, C6, C16, or at other sites. Brecher and Wotiz *(3)* and Korenman *(4)* documented the decrease in protein-binding activity for uterine proteins, which roughly paralleled the decrease in rat uterotropic activity for these estrogenic derivatives. Nearly 20 years ago, Hisaw and his group reported that 16-hydroxylated C18 estrogens, such as estriol and its epimers, when co-administered to spayed rats, largely blocked the uterotropic activity of estradiol in a dose-dependent manner *(5);* extending these observations, Wicks and Segal *(6),* Velardo and Sturgis *(7),* and Huggins and Jensen *(8)* delineated a group of 6-OH and 16-OH steroids, which Huggins and Jensen *(8)* described as "impeded" weakly uterotropic estrogens, capable of competing with and reducing the activity of diethylstilbestrol, estrone, or estradiol in uterotropic activity. In later work, 2-OH estrogens such as 2-OH estrone were also classed among the impeded estrogens *(9)*. Recently, the molecular basis of competition between estrone or estradiol and the impeded estrogens was clarified by the demonstration of the inhibitory effect of the latter on protein binding on uterine estrogen receptors, which can be partly or largely occupied by estriol or other hydroxylated derivatives, preventing the binding and initiation of the usual estrogenic response on DNA, RNA, lipid, and enzyme metabolism stimulated in the uterus by estradiol.

Anticarcinogenic Activity of Estriol

We reported that estrogen carcinogenicity for the breast has thus far only been demonstrated for the most highly active protein binding natural and synthetic estrogens—*i.e.,* estrone, estradiol-17β, diethylstilbestrol, hexestrol, and equilinin *(10)*. None of the C2, C6, or C16 oxidized derivatives of natural estrogens such as estriol have as yet been shown to have any carcinogenic activity for either breast or uterus. Several groups, using different chemical methods for estrogen excretion analysis, have reported subnormal excretion of estriol in about 25% of noncancerous, premenopausal Caucasian women and in over 60% of women with chronic fibrocystic disease of the breast and untreated breast carcinoma. They provided a

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[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

[3] Supported by Public Health Service grants CA 10626-01-02 and CA 080-21 from the National Cancer Institute.

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foundation for *our hypothesis of the human anticarcinogenic function of estriol as a physiologic competitor of estradiol for mammary protein receptors tending to reduce the normal estradiol-induced risk of later mammary carcinogenesis (11).*

Further reviewing the epidemiology of human breast cancer, Cole and MacMahon supported our observations of the inverse correlation between utimate risk of mammary carcinogenesis and known variation of estriol production in various races and at different times in women's lives, such as during pregnancy, castration, and menopause *(12).* They added emphasis upon estrogen production rates during the first 10 years of menstrual function as probably the most critical period for future mammary carcinogenesis. At the present time, all studies of estrogen excretion in cancer patients have been performed decades after the critical period for mammary cancer induction as envisioned by Cole and MacMahon, if indeed an imbalance between estradiol and estriol is a major etiologic factor. Pregnancy and menopause in particular undoubtedly modify estrogen production rates in later years (table 1). The chief endemiologic evidence providing support to this thesis is, however:

Table 1.—Clinical syndromes associated with altered estriol/estrone+estradiol-17 β excretion

a) "Subnormal" estriol excretion:
- 20–25% of normal Caucasian females
- 62% of fibrocystic disease patients
- 62% of untreated, nonstressed breast carcinoma patients, with or without metastases
- Hyperthyroidism

b) Elevated estriol excretion:
- Hypothyroidism
- Menopause (spontaneous)
- Castration
- Pregnancy
- Androgen therapy
- Estrogen therapy
- Glucocorticoid therapy
- Adrenocorticotropic hormone therapy, stress, postoperative state

a) The most significant protection against future breast cancer is early pregnancy, before 25 years of age, with its attendant 1,000-fold increase in estriol production, compared to only a 100-fold increase in production of ovarian estrogens *(13).*

b) Castration performed early in reproductive life seems more effective in reducing subsequent risk of breast cancer than that performed late on premenopausal or menopausal women *(12).* Breast cancer has not, to our knowledge, been reported in chronic adenohypophyseal insufficiency with amenorrhea *(14),* but it does occur occasionally in women bilaterally oophorectomized 2–20 years before recognition of disease. Removal of the ovarian source of estradiol production impairs slightly, if at all, continued estriol biosynthesis throughout life, derived largely from peripheral metabolism of adrenal-secreted Δ-4-androstenedione to estrone and thence to estriol, independent of ovary or adrenals. Adrenocortical dehydroepiandrosterone (DHEA) biosynthesis probably provides part of the precursors for Δ-4-androstenedione biosynthesis, since stress and adrenocorticotropic hormone (ACTH) therapy markedly increase estriol excretion quotients *(10).*

c) Low estriol excretion has not yet been reported in Japanese populations, in whom breast cancer risks are notably low. In Caucasians, only hyperthyroidism has been associated with reduced estriol excretion, in addition to precancerous and cancerous breast lesions *(11).*

d) Subnormal excretion of estriol in fibrocystic disease and in breast cancer before endocrine therapy has been observed by some investigators. Schweppe *et al.* showed that subnormal estriol excretion in postmenopausal breast cancer patients became qualitatively more subnormal after 3-day ACTH therapy, instead of rising as it did in the age-matched controls without cancer *(15).* Schubert, Bacigalupo, and Karlapp observed reduced conversion of estradiol-17β to estriol in patients with chronic fibrocystic disease and with untreated mammary carcinoma, compared to healthy age-matched controls *(16–18).*

Inhibition of Induced Mammary Cancer by Estriol

To supplement the above evidence, we now offer further data on two aspects of the anticarcinogenic action of estriol:

a) After 120–500 days' observation, no breast tumors developed in any Sprague-Dawley intact female rats in whom 700–1200 μg of estriol had been serially implanted every 2 months. One fibrocystic nodule appeared in one control rat. Still being observed are 18–60+ rats in each group.

b) Some 25 experiments were performed during this same period with the use of littermates fed 20 mg 7,12-dimethylbenz[*a*]anthracene (DMBA) in sesame oil at 45–50 days of age. About 62% of the DMBA-fed female rats developed mammary tumors, ranging from hyperplasia of the duct system, through adenocarcinoma, to undifferentiated or scirrhous carcinoma (tables 2, 3). Implanting estriol pellets within 24–48 hours of administration of DMBA inhibited carcinogenesis, with an 80% reduction of palpable mammary tumors induced by the carcinogen. The difference observed was significant at $P = 0.001$. Implanting DHEA pellets did not alter the induction of breast tumors by DMBA. Estrone and estradiol implantation also reduced tumor formation. Lacassagne, Jayle, and Hurst reported that estriol, but not estrone or estradiol, completely inhibited hepatic carcinogenesis induction by *N,N*-dimethyl-*p*-phenylazoaniline (butter yellow) in Sprague-Dawley females *(19)*. Thus far, estriol administration has not visibly affected the growth rate of established tumors in our rats, in any of our experiments, however.

Table 2.—Induction of breast tumors by DMBA in intact Sprague-Dawley females (120–500 days of follow-up)

	Number of tumor-bearing rats	
	Total surviving 30 or more days	Total rats with tumor (%)
Controls, no therapy	0/27	0
DMBA at 45–50 days	38/61	62
DMBA + estriol	5/42	12*
DMBA + estradiol-17β	5/23	22†
DMBA + DHEA	5/9	56
Estriol controls	0/36	0
Estradiol-17β controls	0/4	0
DHEA controls	0/6	0

*0/13 animals pretreated with estriol 48 hours before DMBA.

†5/18 animals pretreated with estradiol-17β 48 hours before DMBA.

Estriol Excretion as a Predictor of Clinical Cancer Remission

Does the estriol excretion of breast cancer patients offer any prognostic aid in selecting the appropriate type of hormonal therapy for established breast carcinomas? There are no available prospective studies that provide critical data on this point.

In 1958, Bulbrook and his associates reported some preoperative estrogen-excretion studies of a few patients. These studies indicated little difference in calculated means of the ratio of estriol to estrone and estradiol in the urine between responders and nonresponders to ablative surgery (table 4) *(20, 21)*. In 1963, Jull, Shucksmith, and Bonser were among the first to use these estriol excretion quotients to correlate estrogen excretion with subsequent response to adrenalectomy *(22)*. They used Brown's 1955 estrogen analytic method (as did Bulbrook's group in part), which seriously underestimated estriol. Of 22 patients deemed to respond well to subsequent adrenalectomy, 9 preoperatively excreted subnormal estriol quotients. Of 18 patients who failed to respond to adrenalectomy, only 3 preoperatively excreted what appeared to be subnormal estriol quotients by our definition, 0.7 for premenopausal and 1.0 for post menopausal women *(11)*. The median value for the quotient of the responders was somewhat less than that of the nonresponders. The latter patients indeed might have had tumors dependent on prolactin-growth hormone, rather than tumors initiated by estrogen.

Table 3.—Induction of breast tumors by DMBA in castrate Sprague-Dawley females (100–500 days of follow-up)

	Number of tumor-bearing rats	
	Total surviving 30 or more days	Total rats with tumor (%)
Controls, no therapy	0/11	0
DMBA at 45–50 days	0/23	0
DMBA + estriol	1/18	6
DMBA + estriol + estrone	2/18	12
DMBA + estriol + estradiol-17β	1/10	10
DMBA + estrone	0/6	0
DMBA + estradiol-17β	0/3	0
Estriol	0/21	0
Estradiol-17β	0/9	0

In our own report of some 60 patients receiving various types of therapy for breast carcinoma, estriol excretion was measured solely by Brown's 1957 improved method. Nearly all of the small number of objective responses noted with castration, androgen, estrogen, or adrenal corticoid therapy occurred following a return to normal in the measured estriol quotient (mg estriol/24 hours)/(mg estradiol/24 hours + mg estrone/24 hours) *(11)*. However, these therapeutic measures all raised the relative quantity of estriol, compared to that of estrone or estradiol in the urine, from the subnormal value found in 62% of breast cancer cases. Thus there may be just fortuitous association of two independently related processes.

Among our patients responding to castration, sex hormones, or glucocorticoid therapy, 3/13 estriol quotients were subnormal, with a median value of 2.5; 10/20 nonresponders were initially subnormal, with a median estriol quotient of 1.1. No statistically significant association between pretreatment estriol quotient and endocrine-induced remission was established (table 4).

TABLE 4.—Estriol excretion of patients in relation to subsequent remission induction by ablative or administrative hormone therapy

Author and reference	Therapy	Total patients with and without remissions	Estriol, μg/24 hours	
			Estrone + estradiol-17β, μg/24 hours	
			No remission	Remission
Bulbrook *et al.* (*20*)	Adrenalectomy, hypophysectomy	11		1.09 (0–4.8)*
		8	0.80 (0–2.0)*	
Jull *et al.* (*22*)	Adrenalectomy	22		0.90 (0–10)*
		18	1.55 (0.50–00)*	
Lemon *et al.* (*11*)	Castration, sex hormones, glucocorticoids	13		2.5 (0.1–4.2)*
		20	1.10 (0.1–7.9)*	

* Median (± range).

Tumor Protein Binding of Estriol and Estradiol in Hormone-"Sensitive" Tumors

To further document whatever therapeutic effect estriol might possess on hormone-dependent neoplasms, we investigated the inhibition of tritiated estradiol binding to surviving tissue slices of DMBA-induced tumors in our Sprague-Dawley, carcinogen-treated rats. The tumors were excised, along with diaphragm and uterus, before and after oophorectomy (table 5). Thin slices of tumor were incubated in Krebs-Ringer-phosphate medium containing 0.5% human serum albumin by the procedure of Terenius (*23*). Sections to be tested were incubated for 1 hour at 37°C in nonradioactive DHEA (3.3×10^{-7} M), estriol (3.2×10^{-8} M), and estradiol (3.3×10^{-8} M). Then approximately 30,000 cpm of 6,7-^{3}H-estradiol-17β was added (4.5×10^{-11} M), and 1-hour additional incubation was carried on. The slices were then transferred to fresh buffer substrate containing cold estradiol (3.3×10^{-8} M) and incubated at 25°C for 1 hour more. They were removed, weighed, ultrasonically vibrated in 0.5 N NaOH for several hours, and acidified with 10% trichloroacetic acid (TCA) to precipitate all residual proteins. The radioactivity of the clear supernatant portion was measured to 4,000–6,000 counts per 40 minutes in a Unilux Model II scintillation spectrometer.

The muscle controls show some plus-and-minus variation in tritium binding, usually within a 5–10% range/unit weight, for the DHEA, estriol, and estradiol preincubations, compared to slices not preincubated with nonradioactive steroids (table 5). The uterus showed a 20–50% reduction of bound tritium, soon and late after castration, with the least reduction of ^{3}H-estradiol binding before castration. The breast tumors, which were of variable morphology and responded differently to castration, showed the most consistent alteration of tritium binding soon after oophorectomy. Estriol was only slightly less effective than nonradioactive estradiol in blocking by about 20–40% the subsequent tissue binding of ^{3}H-estradiol. DHEA was least active in "sensitive" tumors. Tumors rapidly regressing after castration usually fell into the "sensitive" group, whose ^{3}H-estradiol binding was inhibited over 10% by preincubation with nonradioactive estradiol. Tumors not responding to castration usually did not have receptor proteins, as judged by estradiol preincubation analysis.

Mammary-tumor estrogen receptors may therefore differ somewhat from uterine estrogen receptors in these respects:

a) Estriol appears to be more nearly comparable to estradiol in binding to receptor proteins *in vitro*.

b) Tumors, which have been acutely deprived of ovarian steroids and which have not yet undergone marked atrophy or necrosis, appear to be most useful for demonstration of ^{3}H-estradiol uptake and its inhibition by preincubation with estriol. After a period of estrogen withdrawal, exposure of surviving tissues to estrogens sometimes may induce

TABLE 5.—Inhibition of 6, 7-^{3}H-estradiol-17β binding to tissue slices of rat breast tumors induced by DMBA by preincubation with DHEA, estriol, and estradiol-17β

	Total No. tumors/rats	Mean uterine weight (g)	Control cpm/mg wet weight	Percent change after incubation with:		
				3.3 × 10^{-8} DHEA	3.2 × 10^{-8} estriol	3.3 × 10^{-8} estradiol
A. Hosts castrated 1–10 days with "sensitive"* tumors:	12/8	0.36				
Uterus			6.0	−2	−15	−36
Diaphragm			3.1	−10	−1	0
Breast tumors			4.4	−15	−22	−32
B. Hosts castrated 1–10 days with "resistant" tumors:	7/7	0.40				
Uterus			4.1	+3	−5	−28
Diaphragm			2.3	−6	+3	−3
Breast tumors			2.0	+8	+27	+39
C. Intact hosts†	5/5	0.80				
Uterus			2.2	−6	+12	−15
Diaphragm			3.1	−3	+3	−1
Breast tumors			4.1	−4	−3	+1

* "Sensitive" tumors are those showing over 10% reduction of (6, 7)^{3}H-estradiol-17β binding by preincubation with estradiol-17β.

† All tumors included.

an *increase* in subsequent capacity to bind tritiated estrogens in hormone-insensitive tumors—a change which has not, to our knowledge, been reported in the uterus and which did not occur in the uteri in this study.

Somewhat similar methods of inhibition analysis of estrogen binding were carried out in tumor homogenates by the method of Brecher and Wotiz *(3)*. However, in addition, 0.05 M cysteine was used to stabilize receptor protein sulfhydryl groups against oxidative activity during incubation. Tumor homogenates were rapidly prepared in a VirTis homogenizer at 0°C and quick-frozen and stored at −70°C. These incubations, in general, confirmed the tissue slice studies; but the change in radioactivity in the supernatant after incubation was relatively slight and variable. Both estriol and estradiol preincubation inhibited by about 1–3% the binding of ^{3}H-estradiol-17β to the 500 × g sedimentable fraction of the homogenates (table 6).

This technique was also applied to several biopsy samples from humans with fibrocystic disease of the breast. Even more marked changes in the binding of estradiol were observed, after estriol preincubation at 10^{-6}–10^{-8} M concentrations of steroid (table 7). We, therefore, believe that the observa-

TABLE 6.—Inhibition of 6, 7-^{3}H-estradiol-17β binding to homogenates of rat breast tumors induced by DMBA by preincubation with DHEA, estriol, and estradiol

"Sensitive"* tumors		% change after incubation with:			"Resistant" tumor		% change after incubation with:		
Tumor†	%^{3}H-E$_2$ in pellet‡	DHEA	Estriol	Estradiol	Tumor†	%^{3}H-E$_2$ in pellet‡	DHEA	Estriol	Estradiol
R25B(+)	29.6	−0.2	−1.6	−3.1	R23A(−)	33.3	+0.7	+0.6	+0.7
T6B(+)	32.0	−1.3	−1.3	−0.7	T6A(−)	36.0	−0.9	+0.1	+1.9
H9A	35.8	−1.5	−1.4	−4.3	T6A(−)	41.3	+3.2	+0.5	+1.1
Q1	26.8	−0.6	−1.3	−2.0	R22(+)	41.4	−1.3	+0.5	+0.1
R24(+)	35.5	−0.6	−2.1	−1.0	G25	24.3	−0.4	−0.4	+2.5
I8(−)	36.0	−2.9	−3.3	−0.9	G25	30.6	+3.1	+3.0	+6.2
N1	42.2	−1.7	−0.9	−0.7	H9	46.8	−1.0	−1.3	+0.9
					L4	27.4	−2.0	−0.3	+0.8
					L4	32.2	+2.1	+2.3	+1.5
					I11	15.7	−1.0	+1.2	+1.1
					Mean	32.9	+0.3	+0.6	+1.7

* "Sensitive" tumors are those showing over 10% reduction of 6, 7-^{3}H-estradiol-17β binding by preincubation with estradiol-17β.

† From tissue slice experiment: (+) indicates tumor classified as "sensitive"; (−) indicates tumor classified as "resistant."

‡ Percent ^{3}H-estradiol in pellet refers to proportion of radioactivity initially added which sedimented with centrifuged protein precipitates in controls without prior steroid exposure.

tion made on induced mammary tumors of rats may later be applied to an extended study of the competitive aspects of inhibition of estrogen binding to breast biopsies in benign and malignant disease of man, to predict hormone-responsive patients.

TABLE 7.—Change in bound 6,7-^{3}H-estradiol-17β in human breast homogenates after preincubation with estradiol, estriol, DHEA, and testosterone

Patient	cpm tritiated estradiol-17β added	cpm recovered in precipitate after 500 × g	Steroid preincubated	cpm recovered in precipitate after exposure to 3.3×10^{-8} M steroid	% change in radioactivity
P.	10,349	1620	Estradiol	1161	—28
			Estriol	747	—54
			Estradiol-17α	934	—43
	9889	1941	Estradiol	1749	—10
			Estriol	1591	—18
			Estradiol-17α	1684	—13
L.B.	10,077	2098	Estradiol	1925	— 8
			Estriol	1885	—10
			Testosterone	1758	—16
			DHEA	1723	—18
	10,186	2202	Estradiol	1989	—10
			Estriol	2187	— 1
			Testosterone	2002	— 9
			DHEA	1947	—11
M.N.	10,262	1407	Estradiol	1015	—28
			Estriol	1146	—28

Clinical trials of estriol have now been initiated in advanced breast cancer, with the use of daily doses of 2.5–10.0 mg in 8 patients. In 2 of these patients, there has been convincing clinical evidence of a rapid exacerbation of their bone pain. This necessitated hormone withdrawal in both patients, and one of these patients had to be hospitalized. Thus human breast tumors sometimes do have estriol-binding proteins, which may initiate augmented tumor growth. Two other patients, who had no evidence of tumor-endocrine dependence by any other clinical test, tolerated 2.5–5.0 mg estriol for up to 2 months, but there was no clear-cut remission or exacerbation.

CONCLUSIONS

The current available evidence relative to the predictive value of estriol production and/or excretion rates pertains chiefly to its apparent anticarcinogenic effect, perhaps mediated in part through its capability of competing with estradiol for protein estrogen receptors in the uterus and breast. We reported in this study observations of estriol inhibition of estradiol-17β binding to surviving tissue slices and tissue homogenates of breast tumors. Estriol and estradiol but not DHEA markedly inhibited DMBA carcinogenesis in the breast of female rats, whether given shortly before or after the carcinogen. Therefore, our hypothesis of the anticarcinogenicity of estriol reported in 1966 receives some support.

Once cancer has been established and is invasive, no convincing evidence has been obtained thus far, either experimentally or clinically, to indicate that endogenous estriol production or exogenous administration has a major inhibitory effect on cancer growth.

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DISCUSSION

Chairman: With our crude clinical tools, we can pick patients on the basis of clinical evidence with about as much success as we can by more sophisticated biological means. We can also select more precisely the failures and perhaps prevent these people from going through unnecessary operations and submit them earlier to other forms of therapy, probably chemotherapy which most likely acts in the absence of hormone sensitivity—if there is such a thing as hormone sensitivity.

Dr. Hall: If you have a highly restricted pathway and you compare tumor tissue with normal tissue, does this mean that you can exploit it? To be specific, if there is a rather low fraction of glucose-6-phosphate dehydrogenase or glucokinase, can one conclude this is a narrow pathway, the tumor is highly dependent upon it, and the tumor will die? Or does this mean this pathway is being deleted and is unimportant? They represent two totally different therapeutic implications. The contrary part of the question is the situation you have with a wide-open pathway. In breast cancer does this tend to make you want to exploit this by trying to hit a wide-open pathway, or do you make another assumption and conclude that this is too wide open—there is an excess of enzyme, an excess of the pathway, and it is going to be hard to tackle?

Does this lead you to believe that any test which would separate breast cancers into hormone-sensitive and hormone-resistant would split the whole group of patients into two equal halves? The presence of the binding protein would not guarantee a response; it would merely permit it. In about half of the 50% who had the protein or 25% of the whole group, responses would occur.

Dr. Bulbrook: Is this business of being able to pick up the failures related to this multiple pathway—endocrine pathway business? Maybe the people we have with positive receptive sites or positive discriminants who still fail to respond are dependent on something. Possibly, we have one group buttoned up by failures; if we spread the hormone net a bit, we might be able to pick these up.

As to your next point, about whether there is anything the clinician can recognize, I do not know about the clinician, but it looks to me as if the biochemist can pick up a lot of other things. For example, we are getting reports that tryptophan metabolism is abnormal in breast cancer and the immune response to tuberculin is abnormal. From our data, all these appear to occur in the same patient; so possibly we are starting to pick up a great mass of parameters that additively would make it 100% certain that a patient would be a failure rather than to have the slightly uneasy feeling we have now that we are not operating on someone who should be operated on.

Dr. Jensen: I would like to comment on this failure of people with positive patterns because our interpretation is just a little different from that of Dr. Bulbrook. I mentioned in my talk we do not worry too much about the fact that someone with positive patterns still would not respond. First of all, if you do have a false positive you do not decide not to initiate therapy that might help the patient. Therefore, you adrenalectomize anyway so that you really have not lost anything. Although you would expect that there might be some patients with positive patterns who would fail, you should not expect, if there is any relation between receptors and response, to have ones with negative patterns that will succeed. The reason is that it is likely in a human as well as in the animal that, when you have a metastatic widespread cancer, you will have mixed metastases—some of which have receptors and some of which do not.

I think you can say that there are three possible situations in a metastatic cancer: 1) those in which all the growths are positive, all tests are positive, and the response will be a remission; 2) those in which all the metastases are negative, all tissues give negative test results, and are a failure with adrenalectomy; 3) some that are mixtures; certainly this is true in animals as well as in prostatic cancer patients. Dr. Huggins has shown very nicely that, with multiple skin lesions after orchiectomy, all of them may go away but one. Actually what test result we get depends on which tissue sample the surgeon picks to give us. We did have one case in which the surgeon gave us 3 different samples and all were negative. In addition to the case of this patient, we have only had 2 samples from a single patient. The two samples have always agreed, but still there is the possibility that a patient could have both types of tissue and the samples would not be representative. Thus, if we have mixed metastases, no matter what test result we get, we will expect failures and remission only when they are all positive. Hence we do not worry too much about the positives; we have two of those in our series so far.

Dr. Lemon: We should not overlook the fact that some breast cancer tumors will respond to adrenalcorticoids without adrenalectomy. Samuel Taylor was one of the first to show this. Of patients who did not respond to castration or to sex hormone therapy, 28% of our patients had good remissions on corticoids. This I do not understand. I do not believe anyone has shown that there is any interaction with the estrogen receptors that would explain the antiestrogenic activity of glucocorticoids. Very unlike sex hormone therapy, we have had the same 50% response to corticoids with local, osseous, or visceral metastases.

Dr. Hilf: What we have done to date is to try to identify which pathways seem to be either wide open or perhaps shut down and whether changes in these pathways now correlate with changes in the growth patterns of these tumors. This is due to the fact that sometimes we can elevate the activity of an enzyme and slow down tumor growth and sometimes we can take the same enzyme and show that it has decreased in activity and the tumor has decreased in growth. Glucose-6-phosphate dehydrogenase is the enzyme. If one can identify these pathways and assume that they are critical to the continued growth of the cells, then these would be the logical tools to examine or find inhibitors or stimulants. If we would like to bring α-glycerophosphate dehydrogenase back into as normal range as possible, then we might look for substances that could reverse the apparently inhibited situation of this enzyme.

Dr. Nickson: I would make a strong plea for those essayists who included human data to realize that the strictures on time would not permit them to define and describe with some precision what they mean by "remission," what they mean by "indifferent," and what they mean by "failure." This does vary from institution to institution. It is crucial to have these conditions defined. I do have a question for Dr. Jensen: I was very much taken by the data you presented. Are you not falling into the trap of using your model before you have demonstrated that your model is a real one? That is, you said the surgeon is beginning to use your positives and negatives as determinants of whether or not he or she will operate, and this, to me, erodes the likelihood that you really get a firm test of your model.

Dr. Jensen: You are certainly right. Any patient on whom the surgeon decides not to operate does not come into our series because the clinical results are not available. This has been true in the 6 patients whom I mentioned here. These were all patients on whom the surgeon was doubtful about operating because they were all very sick and thus poor surgical risks. Our data might have been just what made the surgeon decide not to operate. We need more cases before we can dogmatically say to the surgeon: "Don't operate on the negative ones." From our own selfish standpoint, we would rather see a patient operated on so that we will have him available for our series. The ones who are not operated on do not affect our model at all because they are not under consideration. I listed those 6 patients only to show what proportion of negative and positive ones we have.

Dr. Nickson: But they do affect your model if you exclude them.

Dr. Bulbrook: We have precisely the same problem. We got around it by having every patient examined by an independent physician before they come anywhere near to any of the clinical trials. The patient is excluded on the physician's say-so, or if he lets him in, then the protocol is followed. Otherwise, you have the dreadful dilemma of handpicking the people (and of institutes quarreling for the next 20 years), and it means that nobody else can handpick in the same way.

Dr. Taylor: Have we had any correlation between picking patients for estrogen treatment?

Dr. Jensen: So far we have not, but recently the Department of Medicine has become interested in this and they promised us we can have a sample, when it is accessible, of a metastatic growth of patients being treated with estrogen, androgen, or estrogen–progesterone combinations.

Dr. Lemon: Is the receptor protein in human breast cancer possibly an inducible protein? Could it be induced by diethylstilbestrol therapy?

Dr. Jensen: I do not know. We know most about the receptor proteins in the rat, and all we know is that they are at their highest level in the immature rat and the content of the uterus goes down as the rat gets older, partially, I believe, because the uterus grows under estrogen stimulation faster than the receptor is synthesized. There is evidence in the rat that there is a secondary effect of the ovarian secretions on the receptor synthesis. If one determines in a mature rat its total 8S protein receptor content by the height of the 8S peak, one gets a value much lower than in the immature rat. This is mostly because the endogenous estrogen is sitting on these receptor sites and it does not exchange well in the cold. For the first week after ovariectomy, the apparent content of receptor will rise to almost double the value in the intact animal. This we believe is depletion of endogenous estrogen. When it reaches the peak, it is not as high as that in the immature rat per milligram of tissue. Then over the next 7 weeks the receptor content decreases progressively, suggesting that something from the ovary is necessary for the maintenance of the ability of the uterus to make this protein.

Dr. Zelen: First, do the people who have a remission survive longer than the people who do not, and how much longer do they survive? Second, there has been discussion about animal models, and Dr. Bulbrook has made some unfavorable comments about animal models. It seems to me that one of the fundamental problems in human breast cancer is understanding the process of metastases. I would like to know of what value some of the animal models are if they do not metastasize and how this is related to whether one is using a carcinogen-induced tumor or a spontaneous tumor.

Dr. Hilf: I will not defend Dr. Bulbrook in his feeling about animal models. We obviously do not have, at least to my knowledge, ideal models in the animal, but what we have been attempting to do has been to find out how any one of these neoplasms in animals may resemble any type of human disease. It is the type of question that says you have a model and I have a model, and you like working with yours and I prefer ours—but why? We do not have exact answers. We would like to do more work with human carcinomas to determine whether any of the biochemical characteristics of the metastatic lesion differ from the primary. We have about 50 cases in the series and, unfortunately, many of these patients will probably come back with metastases. Hopefully, we can get some tissues from these metastases and compare them with the tissue of the primary lesion. We have seen very little difference in the biochemical characteristics of the human carcinomas in patients who present initially with metastases, so that they may not be significant. Often with the models, it is so long before metastasis occurs that the animal dies from its primary transplanted tumor before the time of metastasis.

Participant: I believe it has been shown that metastases can be produced from 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced tumors if cells of the tumors are injected into the portal vein. I think this was reported in Dr. Dao's laboratory. It might be possible to produce a metastasized model in that way.

Dr. Dao: This is only what I have read. I have not done such experiments.

Dr. Hilf: I am impressed though, in the DMBA-induced tumor, how often there is invasion of skin, and dissecting these out is not simple. Perhaps Dr. Dao would like to comment. These behave as malignant tumors do in many cases by local tissue invasion.

Dr. Dao: One reason, of course, is probably the strong estrogenic patterns of these tumors, this being probably a factor in which you prevent an appearance of actual metastases. We do find tumor cells in the spleen in DMBA-induced tumors. A few years ago we had established a model for producing metastases fairly easily, with bone and liver metastases.

Dr. Heidelberger: We are talking today about two different tests. One is the large clinical and laboratory screening of what goes on in steroid metabolism. Dr. Jensen has shown very conclusively and very elegantly in past years that this estrogen receptor protein is of primary importance in the mechanism of action of estrogen at the biochemical, cellular, and molecular level. We have what seems to be a very firmly established biochemical mechanism of action and now he is applying that to tests to see whether it can be correlated with clinical predictions. I seem to have sensed, perhaps incorrectly, some hostility here on the part of some clinicians towards Dr. Jensen's tests, in the way that they are being conducted. But at least the tests are based on a very firm biochemical and molecular rationale.

PANEL III

IMMUNOLOGIC FACTORS

Preclinical Evaluation of the Interrelationships Between Cancer Chemotherapy and Immunity[1, 2, 3]

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THE CHEMOTHERAPY of neoplastic diseases has achieved some success, in that a small percentage of patients with certain types of cancer have been brought into complete remission by the use of drugs, and are free of detectable disease 5 years or more after diagnosis *(9, 31, 41)*. In fact, in certain instances, choriocarcinoma in women, Wilms' tumor, Burkitt's lymphoma, and acute lymphocytic leukemia appear to have been eradicated. In other instances, although cures have not been achieved, induction of complete remission and significant prolongation of survival have been attributed to the effects of drugs.

Despite continuing progress in the application of pharmacological knowledge to the treatment of cancer, substantial obstacles need to be overcome before chemotherapy becomes widely useful in the definitive management of neoplastic diseases. It is generally accepted that all cancer cells in the host need to be killed to achieve cures. In most cases, this aim cannot be readily reached by use of the relatively nondiscriminating, antiproliferative, and cytotoxic drugs developed to date.

Evidence obtained in animals *(45, 66, 90)* and man *(24, 44)* indicates that primary tumors have antigenic characteristics not present in normal tissues of the adult host. Therefore, hopefully, specific immunological responses against tumors can be exploited therapeutically. The potential usefulness of the immunological approach to the treatment of neoplastic diseases resides primarily in the intrinsic specificity of the immunological response *(90)*. Consequently, attempts are being made to develop clinical cancer immunotherapy following various types of experimental leads *(5, 51, 72, 88, 89)*. It may be possible to achieve a restoration of the immunological response, a breakage of tolerance, a reduction of enhancement, and immunotherapy based on either the transfer of sensitized cells or of specific cytotoxic sera, or the active specific or presumably nonspecific stimulation of the host to tumor antigens.

Although conceivably effective immunotherapy may achieve the desired degree of selectivity against neoplastic cells, considerable difficulties need to be overcome before tumor immunity can be exploited therapeutically. For instance, specificity of antitumor effects cannot be readily achieved when so-called passive immunotherapy is established by the transfer of heterologous immune serum or cells. Immunological enhancement may develop as a result of active immunization with tumor cells or cell fractions. A successful nonspecific stimulation of the host response against neoplasia must overcome the intrinsic inefficiency of the immunological reactions to primary tumors. Usually, the defenses of the host are rather ineffective against large and widespread tumors. Thus, in mice, immunity elicited against 3-methylcholanthrene-induced autochthonous tumors is overcome by the use of sufficiently large numbers of cells *(46)*. *In vitro,* lymphoid cells from cancer patients were shown to react against autochthonous tumor cells *(29, 30)*. Some reactivity of human peripheral leukocytes and serum against autochthonous tumor cells was also demonstrated *in vivo (84)*. Therefore, the inefficiency of the host's response to its own tumor would seem to have a quantitative, rather than a qualitative, basis. Such intrinsic inefficiency of the immunological response to established autochthonous tumors may be one of the major obstacles in any attempt to exploit tumor immunity therapeutically.

Despite the current limitations of both cancer chemotherapy and immunotherapy, a combination of these two approaches may eventually lead to a cure. Conceivably, having been adequately reduced in size by chemotherapy, tumors may be eradicated by existing immunity or by immunotherapy. In-

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[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

[3] Supported in part by Public Health Service research grant CA 11047 from the National Cancer Institute.

deed the apparent cures of certain types of cancer, presumably obtained by chemotherapy, may represent inadvertent examples of a cooperative action of drugs and existing immunity.

Attempts to combine chemotherapy and immunotherapy may be difficult because most anticancer drugs also inhibit the immunological responses to various types of antigens *(6, 21, 37)*, probably by virtue of the very antiproliferative action which is at the basis of their antitumor effects. Reduced therapeutic activity may be seen with drugs which depress the defenses of the host at doses effective against the tumor *(77)*. Obviously such a self-defeating drug action may also reduce the effectiveness of attempts to combine chemotherapy with immunotherapy. This danger may be particularly real in those cancer patients in which the immunological responsiveness is already reduced as a consequence of the disease.

Therefore, the potential usefulness of a chemotherapeutic treatment is in parallel with the degree of antitumor selectivity of such treatment—selectivity considered with reference to not only such gross antiproliferative toxicity as that causing bone marrow and gastrointestinal damage, but also to more subtle effects on the immunological responses. Consequently, it seems important to assess both the danger that antitumor drugs may depress the immunological reactions to tumors and the alternative possibility that selective treatments with such drugs may completely or partially spare the immunological responsiveness and thus may act cooperatively with residual antitumor immunity or with combined immunotherapy.

At present, without sufficient direct information on the effects of anticancer agents on host defenses directed against autochthonous tumors in animals or man, at best only circumstantial evidence is available for a general consideration of the possible relationships between cancer chemotherapy and tumor immunity. The opposite and contradicting implications of this information will be discussed with the use of relatively few examples selected from the extensive data reported in the literature, data which have been thoroughly reviewed in recent years by others *(6, 7, 21, 37, 40, 78)*. The possible usefulness of certain experimental systems in assessing the potential cooperative action between drug treatments and immunity will be also considered.

Information Consistent With a Possible Depression of Responses of Hosts to Tumors by Antitumor Drug Treatments

Relatively little is known about the characteristics of the immunological response to autochthonous tumors in man. Factors, such as the strength of the antigenic stimulation by tumors and the time at which it occurs or the existence and possible fluctuations of a state of tolerance to neoplasms, have not yet been assessed. It is usually assumed that the immunological responses to tumors in man fall at least qualitatively within the patterns derived from studies in animals or from clinical investigations based on the use of nontumor antigens. Thus one assumes that in man the initial immunological reactions to the immunogenic stimulus of the tumor probably have occurred long before the neoplasm is recognized clinically and chemotherapy is initiated. Therefore, with reference to the possibility that antitumor drug treatments depress the host's response to tumors, particularly ominous are those data indicating that anticancer drugs inhibit established immunological responses. Several such examples are mentioned in the literature.

In rabbits, 6-mercaptopurine (6-MP) inhibited an ongoing response to bovine serum albumin (BSA), when antigen was given for 40 days and the drug was injected for 8 days starting on day 28 *(11, 21)*. It was suggested that 6-MP inhibited the participation in the immunological response of cells which were newly recruited under the condition of daily antigenic stimulation *(21)*. Should this hypothesis be correct, then possibly, in cancer patients, antigen-sensitive cells may continuously be recruited during continuous exposure to weak tumor antigens and the proliferation of these cells may be inhibited by a drug at doses effective in the treatment of the target tumor.

In rats, established delayed hypersensitivity to BSA was inhibited by vincristine or vinblastine at lethal doses. Inflammatory reactions were also observed *(4)*. In strain-2 guinea pigs, established delayed hypersensitivity to tuberculin was inhibited by a 2-week treatment with methotrexate (MTX) at doses which did not cause leukopenia, overt lymphotoxicity, or anti-inflammatory effects *(20)*. The response was positive 10 days after the end of drug treatment. Species differences may account for a great deal of variability, however. For instance, in man, MTX had no detectable effects on established delayed hypersensitivity to tuberculin and other antigens at maximum tolerated doses *(36, 74)*.

Delayed hypersensitivity responses are reduced in patients with certain forms of neoplasia, particularly Hodgkin's disease and advanced carcinoma *(3, 26, 37)*. In Hodgkin's disease, the lymphocytes seem to be functionally deficient, as indicated by their impaired reactivity in the lymphocyte-transfer test *(1)* and by their reduced capacity to undergo blastogenesis after *in vitro* stimulation with phytohemagglutinin (PHA) *(39)*. Anticancer drugs may be more effective in depressing delayed hypersensitivity reactions in patients with immunological deficiencies. Consistent with this possibility is the demonstration that in psoriatic patients, who are often immuno-deficient, certain antimetabolites were much more effective than in normal individuals in suppressing the induction of delayed hypersensitivity to dinitrochlorobenzene and *p*-nitrosodimethylaniline and the antibody response to plague vaccine *(49)*. Nevertheless, in these patients, secondary responses to typhoid-paratyphoid vaccine were not impaired by treatments with several of the antimetabolites tested *(14)*.

The inhibition of secondary antibody responses to various antigens by a number of anticancer drugs has been documented in several species, including man *(47, 81, 85)*. This inhibition should be stressed particularly because in animals certain drug combinations were reported to have marked synergistic inhibitory effects. For instance, in rabbits, the concurrent administration of 6-MP and Duazomycin A at tolerated doses completely abolished the secondary response to diphtheria toxoid, while either drug alone had only moderate effects *(71)*. Similar synergisms may also occur in patients undergoing intensive treatments with multiple drug combinations which are currently studied in cancer chemotherapy.

In rabbits given toxic doses of 6-MP for 8–10 days after a secondary stimulation with BSA, not only was the secondary antibody response abolished, but also the tertiary response when the same antigen was given 50 days after completion of the secondary response study *(19, 21, 47)*. Animals that had become unresponsive to BSA still responded normally to unrelated antigens. The data implied that, at the high doses used, 6-MP deleted immunological memory and induced tolerance to BSA. Although the doses of 6-MP used were much more toxic than those employed in chemotherapy, possibly the so-called memory cells are relatively more sensitive to drug inhibition in man than in animals.

Inducing immunological tolerance in adult animals is facilitated by several anticancer drugs *(21, 78)*. This effect is directly related to the amount of antigen present *(80)* and inversely related to the degree of antigenic difference involved; it is also influenced by the type of antigen and the form in which it is presented *(78)*. Facilitation of the development and maintenance of tolerance in man may be one of the real dangers of cancer chemotherapy, particularly because in cancer patients the tumor can be large and the antigenic difference between tumor and host is probably rather small.

Therapeutic effects in established autoimmune diseases have been achieved by use of MTX and 6-MP *(79)*. In patients with leprosy receiving 400–800 mg/day of cyclophosphamide for several months, the symptomatology dependent on the host's response was improved *(12)*. These results could not be confirmed with lower doses of cyclophosphamide *(76)*. The local immunological reaction to the lymphocytic choriomeningitis virus and the consequent death of the infected mice were prevented by MTX *(27)*. Anticancer drugs can reduce an ongoing reaction to renal allografts in man. Thus, actinomycin D is effective in treating the renal rejection crisis which often occurs, despite immunosuppressive regimens, within 30 days after renal transplantation *(2, 64)*. Although peripheral mechanisms, possibly anti-inflammatory in nature, are probably partly responsible for the therapeutic effects of anticancer drugs in the treatment of both autoimmune diseases and kidney rejection crisis, these effects do result from an impairment of established host defenses.

The so-called opportunistic infections often occurring in cancer patients during intensive chemotherapy *(8, 43, 83)* should also be mentioned, since it is generally recognized that these infections are related to a depression of host defenses. Most infectious agents involved are endogenous in origin, which supports the idea that a decrease of preexisting immunity by anticancer drugs is at the basis of the disease. The pattern of infection is also altered in some cases. For instance, patients with acute leukemia undergoing intensive chemotherapy often develop severe infections due to *Candida* *albicans* and Pseudomonas *(8, 34)* or have evidence of cytomegalovirus *(13)*. In some patients, the use of antibiotics may be partly responsible for the change in infection patterns *(82)*. In recent years the frequency of opportunistic infections has increased in relation to the increased use of potent cytotoxic drugs in cancer chemotherapy *(34)*. This is also consistent with the view that the effects of these drugs are causatively related to the onset of these diseases, particularly in patients with leukemias and lymphomas. Following treatment with some of the same drugs, however, opportunistic infections are not seen nearly as often in patients with other forms of cancer. Thus the type of disease may also influence the etiology of the infection.

When given shortly before or shortly after the implantation of the tumor, several anticancer drugs have impaired tumor allograft rejection in mice. Thus MTX, 6-MP, and, particularly, melphalan reduced the rejection of allografts of a subline of L1210 resistant to antifolics in C57BL mice *(42)*. In BALB/c mice, both MTX and melphalan conditioned the progressive growth of this L1210 subline at doses insufficient to prolong DBA/2 skin-allograft survival *(22)*, thus suggesting that a lower degree of immunosuppression is adequate to allow tumor allografts to grow than that required to prolong the survival of nonproliferating tissue allografts. A number of antitumor agents prevented the rejection of Sarcoma 180 allografts which occurs in a high percentage of Swiss Ha/ICR mice fed a vitamin B_6-deficient diet *(59, 61)*. The incidence of takes and the rate of growth of early transplant generations of 3-methylcholanthrene (MCA)-induced tumors increased in syngeneic C57BL mice treated with drugs, such as cytoxan, MTX, and 5-fluorodeoxyuridine, shortly before tumor implantation *(68, 69)*.

Drug-depressant effects on tumor graft rejection were also confirmed inadvertently in man. In fact, 2 patients reportedly died with disseminated neoplasia a few months after they received implants of kidneys from donors who had died of cancer *(50, 52)*. The tumor in the recipients had the same histological characteristics as those of the original tumor in the donor. In both patients, massive immunosuppression had been applied shortly before and after renal grafting. In another case of inadvertent tumor transfer by renal grafting, after the immunosuppressive treatment with azathioprine and prednisone had been stopped, the kidney was rejected *in situ*, but the tumor continued to grow perirenally; only after surgical removal of the rejected kidney did the residual perirenally outgrown tumor regress *(91)*. Nine months later, another kidney was implanted in this patient, and there was no evidence of further metastatic cancer despite full immunosuppression. The fact that perirenal tumor growth continued after the kidney was rejected *in situ* upon discontinuation of drug treatment is consistent with the data obtained in mice *(22)*; it supports the view that a stronger immunological response is required to reject a proliferating tumor tissue than that sufficient to reject a kidney or a skin graft. Since the tumor was eventually rejected after surgical removal of the kidney, then the immunological reaction to the tumor graft most likely became more effective after removal of the antigenic renal mass. In a similar case, a hepatocarcinoma, transplanted with the kidney, remained undetected for 3 years; it became evident as a result of the development of pulmonary metastases *(92)*. After discontinuation of immunosuppression and renal rejection, the metastases increased in size and number for 3 months before regressing completely.

Recently it was reported that the incidence of malignant lymphomas, particularly reticulum cell sarcomas, and other types of tumor, including carcinomas, appears to be higher among kidney transplantation patients treated with azathioprine, prednisone, and antilymphocytic serum than among the general population *(65)*. Should these initial observations be confirmed, they may lead to the conclusion that the increased incidence of tumors seen is directly related to the immunosuppressive treatment given to these patients.

The information discussed in this section strongly suggests that, in cancer chemotherapy, one should be aware of the distinct possibility that reduced therapeutic effects are obtained as a result of the self-defeating immunosuppressive action of the drugs used.

Information Consistent With the Possibility That Antitumor Drugs Act Cooperatively With Responses of Hosts to Tumors

Despite the evidence discussed in the preceding section, at this time it does not seem justified to negate *a priori* the possibility of exploiting chemotherapy and immunity synergistically in the treatment of at least those neoplastic diseases in which the immunological reactivity of the patient is not substantially depressed by the disease itself. In fact, several studies in animals and in man have provided information that such a cooperative action might be achieved.

Pre-existent active immunity is usually not affected by anticancer drugs when given at the doses ordinarily used in chemotherapy. For instance, in patients with leukemia and carcinomas, established delayed hypersensitivity to several antigens and isohemagglutinin levels were not inhibited by treatments with MTX or 6-MP *(20, 48, 74)*. The blast-like transformation of human peripheral blood lymphocytes exposed to PHA in short-term cultures may indicate the immune reactivity of the donor patient. Studies based on this test revealed that the incidence of blastic transformation was reduced from 71% for lymphocytes obtained before initiation of treatment with 6-MP or MTX to 1.5% for cells obtained during therapy *(38)*. When the two drugs were combined, with or without the addition of prednisone, the response was completely abolished in a few days. Nevertheless, even under these circumstances, cell reactivity recurred as early as 3 days after the end of treatment. When 6-MP and MTX were given at doses which did not cause leukopenia, maximum inhibition of lymphocyte transformation by PHA occurred only after 2–5 weeks of therapy *(38)*.

The other study of a series of 16 leukemic patients treated with intensive 5-day courses of 6-MP or MTX revealed that the primary response to bacterial antigens was markedly inhibited as early as 24 hours after the beginning of therapy *(35)*. However, all patients exhibited some responses 24 hours after the last day of treatment and normal responses 72 hours after the end of therapy. Both these data and those on lymphocyte transformation mentioned in the preceding paragraph are compatible with the possibility of eliciting sequential cooperative actions between drugs temporarily depressing the immune response and immunity. This approach was already followed clinically using bacille Calmette Guérin to stimulate the immunological reactivity of the host after the end of antileukemic chemotherapy *(51)*.

In studies of the cellular changes occurring in the regional lymph nodes during the development of hypersensitivity reactions to dinitrochlorobenzene or oxazolone, some antitumor agents were found to inhibit these reactions by blocking the development of sensitized lymphocytes at different steps along the multicellular pathway leading to the formation of the effector cells *(86)*. For instance, cytoxan inhibited the formation of both pyroninophilic immunoblasts and small lymphocytes whereas MTX blocked only the formation of small lymphocytes and not that of immunoblasts. Therefore, treatments with certain anticancer agents may block the immunological response to the tumor at some steps, particularly those involving cell proliferation, without necessarily affecting some processes related to cell sensitization. Should this be the case, following cessation of drug effects, the immunological reaction to the tumor may be greater and more rapid than that occurring without drug treatments.

The information obtained with tumor grafts can be criticized, especially if unjustified attempts are made to extrapolate data obtained with transplantable tumors directly into situations involving autochthonous tumors. However, data obtained with transplantable tumors can be a basis for a discussion of the depression of transplantation immunity by anticancer drugs, under conditions in which the transplanted tissue is proliferating and is also a target of the drug in question.

It has been shown repeatedly that anticancer drugs can depress immunity against tumor grafts only when given either shortly before or after tumor implantation. For instance, this was observed with alkylating agents and antimetabolites in L1210 *(22, 42)* or Sarcoma 180 *(59)* allografts or isografts of early transplant generations of MCA-induced sarcomas *(68, 69)*. When the tumor is sensitive to the antiproliferative action of a drug with immunosuppressant effects, synergism or antagonism between drug and immunity may be seen, depending on the time of drug administration *(62)* or the relative effectiveness of the treatment on tumor growth and immune response *(15, 18, 54)*. Indeed, synergism between antiproliferative drugs and immunity was also demonstrated with the strain-specific L1210 in DBA/2 mice *(55, 56)*.

As a whole, the information mentioned in this section suggests that a certain degree of optimism is permissible in considering the possibility of exploiting cancer chemotherapy and immunity cooperatively. Indeed the general finding that established immunological responses are usually not affected by anticancer drugs at therapeutic doses, the observation that the inhibition of primary responses is usually rapidly reversed upon termination of treatment, and the experimental evidence that delayed hypersensitivity responses may be blocked without affecting differentiation phenomena occurring during the immunological reaction—all support the proposition that possibly the desired synergism may be achieved between anticancer drugs and tumor immunity.

Animal Test Systems of Potential Use in Evaluation of Interrelationships Between Antitumor Drugs and Tumor Immunity

The evaluation of the interrelationships between drug effects on tumor, drug effects on the immunological reactions to tumor, and antitumor effects of the specific immunological reactions is difficult to achieve within each tumor-host system, because it requires the concurrent measurement of antagonistic and synergistic effects and the analysis of drug actions on the various cell types involved in the different phases of the immunological responses. To these complexities, one must add the fact that differences in host reactions to tumors and, as a consequence, of drug effects in tumor-host systems can be expected not only with different tumors but also with the same tumor at various phases of growth or during growth at different sites. The type of immunological response and its strength are indeed related not only to the type of antigen and its intrinsic immunogenicity but also to the dose of antigen and its mode of presentation to immunologically reactive sites.

Attempts to predict the potential interrelation between anticancer drug and tumor immunity in man from studies in animals must also take into consideration species variability. Differences in immunological reactivity have been found not only among species but also between sexes of the same mouse strain *(25)*. Differences in drug action among species are well known and reflect primarily species-specific characteristics in drug disposition and tissue sensitivity. In cancer chemotherapy, it is particularly important to consider possible differences not only in target tissue sensitivity but also in the ratio between the sensitvity of target tumor and that of normal tissues. In fact, relatively minor differences in this ratio may result in major differences in therapeutic effectiveness of antitumor agents. In the consideration of such a ratio with reference to the trigonal relationships among drug, tumor, and immunological system, it is apparent that species differences may occur in terms of drug toxicity not only to the lymphoid tissues as a whole but also to specific cell types participating in the immunological response.

From the preceding considerations, it would seem almost impossible to develop animal systems capable of predicting the potential interrelationships between antitumor drugs and tumor immunity in man. Nevertheless, the limitations to be overcome are similar to those generally encountered in any attempt to establish experimental tumor test systems capable of providing valid information with reference to the clinical studies. Moreover, individual differences among patients may be almost as critical as other differences in reducing the accuracy of predictions based on information obtained under conditions other than the very one for which the predictions are made. Therefore, although these limitations should be kept in mind for a critical evaluation of the results obtained in the laboratory, they should not deter one from developing model systems which may indeed provide information leading to useful applications. Similar to the requirements to be met in studies of potential drug toxicity *(58)*, also in the evaluation of potential antagonisms and synergisms between cancer chemotherapy and immunity, it seems advisable to use a variety of experimental systems under a variety of pharmacological conditions. Conclusions based on data obtained in a multiplicity of relevant model systems have an increased probability of being valid also in comparable human situations.

Relevant investigations, with various tumor-host systems, should comply with the following general criteria: *a*) The effects of drugs should be studied in relation to pre-existent or ongoing immunological reactions to weakly immunogenic tumors; *b*) various regimens of drug administration should be assessed for their capacity to preserve the immune response to tumors or to allow its preferential recovery; *c*) delayed hypersensitivity-type responses and humoral antibodies should both be considered —the antibodies for either cytotoxic or enhancing properties; and *d*) the balance between cell-mediated and humoral antibody reactions to tumors should be evaluated in view of the possibility that it affects the growth of the tumor and the results of chemotherapy *(56)*. Indeed some enhancement of tumor growth, mediated by humoral antibodies, may also occur in man *(29)*.

Optimally, the systems employed should be based on the use of autochthonous tumors, which probably represent the closest approximation to human neoplasms *(90)*. *In vivo* studies with autochthonous tumors have limitations similar to some of those encountered in clinical studies, particularly because in most cases each animal must represent its own control. In fact, the immunogenicity of chemically induced tumors is usually specific for each tumor *(45, 67)*. In so-called spontaneous mammary tumors, cross-reactivity is limited to those instances in which the immunized mice have not been exposed to mammary tumor virus (MTV) at birth. In mice neonatally exposed to MTV, however, specific immunogenicity could be demonstrated for each mammary tumor, possibly because of the state of unresponsiveness to otherwise stronger cross-reacting MTV and MTV-associated antigens *(63, 87)*. Since autochthonous tumors induced by viruses share common virus-related antigenic properties *(14)*, they may provide some operational advantages in that groups of tumors of presumably similar immunogenicity may be studied. *In vitro* techniques are becoming available, however, for the study of the reactivity of lymphocytes to autochthonous tumor cells. Some of these techniques *(30, 33)*, which were applied also in studies of lymphocyte reactivity to human tumor cells, may elucidate some of the possible interrelationships between antitumor agents and tumor immunity. Indeed they may also offer the possibility of the establishment of test systems applicable to studies of the *in vivo* effects of drugs on the balance between cellular immunity and humoral antibody responses to tumors *(29, 32)*.

First or very early transplant generations of primary tumors in isogenic mice offer the advantage that the immunological response of the recipient animals is presumably directed against so-called tumor-specific antigens and that adequate groups of comparable tumor-bearing mice can be readily established from a single donor. Systems based on MCA-induced sarcomas in C57BL mice have been used to study the depression of tumor immunity by cytoxan and other anticancer agents *(68, 69)*. Tumors induced by the Moloney virus have been used to study the interrelationship between chemotherapy and immunity *(10, 23)*. The observation that certain alkylating agents are more effective against first transplant generations of spontaneous mammary tumors of the C3H mouse than against the primary tumors in the original hosts *(75)* is consistent with the possibility that the immunological response to the transplanted tumors was stronger than that against the primary ones and had adjunct effects in cooperation with the drug treatments. The validity of this idea should be confirmed by the exclusion of the alternative possibility, that nonimmunological factors related to tumor transplantation were at the basis of the chemotherapeutic differences observed.

In systems based on established transplantable tumors, it is unlikely that the immunological response is directed primarily against so-called tumor-specific antigens, especially when the tumor has a long transplantation history during which immunogenetic changes probably occured in tumor or host strain. In many cases, however, the transplanted tumors grow progressively in 100% of the hosts, even after implantation of only a few cells, which suggests that the tumor cells are weakly immunogenic or are growing so rapidly that they overcome the immunological response of the host before it is efficient enough *(70)*. The immunological reaction may become effective, however, in eliminating a tumor impaired in its growth by chemotherapy. Under these circumstances, the expression of the host's response in terms of target cell destruction is conditioned by the concurrent or preceding antiproliferative action of effective drugs. Within this frame of reference, certain transplantable tumor systems can be used to study the interrelationships between the effects of antitumor agents and those of relatively inefficient transplantation immunity on weakly immunogenic and/or rapidly proliferating tumor grafts. Cell-mediated graft-rejection mechanisms and cytotoxic or enhancing humoral antibodies can be readily studied in these systems depending on the tumor used. Results consistent with these views have been obtained in this laboratory with Leukemia L1210 *(55–57, 60)* and Sarcoma 180 *(15–18, 54, 56, 59, 61)*.

Leukemia L1210 is an example of so-called strain-specific tumors, in that it grows only in DBA/2 mice and F_1 hybrids or backcrosses derived from them. All DBA/2Ha-DD mice die within

2–3 weeks from the time of inoculation of 10 leukemic cells or less. After the inoculation of 10^6 leukemic cells, the average survival time is 7–8 days. Without drug treatments, the weak response of the host to the tumor is indicated only by the differences in survival curves between nonirradiated and preirradiated mice inoculated with 10–10^7 L1210 cells. The efficiency of the immunological response can be increased if prospective recipients are immunized with X-ray-killed L1210 cells. Following selective treatments with certain drugs, the immunological reaction is effective enough to eliminate residual tumor cells. Thus, complete therapeutic cures are obtained, even though some of the drugs used, such as arabinosyl cytosine, have an immunosuppresive action in the very DBA/2 mouse in which they exert cooperative action with the immunological response to L1210 *(55)*.

The L1210-DBA/2Ha mouse system can be used for a study of not only the antileukemic effects of a drug but also the cooperative action of such a drug and the relatively inefficient immunological response to the tumor. Moreover, sometimes the depression of the immunological response by the drug under examination can be studied. The antileukemic effects of a drug can be studied on early or advanced L1210 according to well-known procedures. The potential cooperative action of drug and immunity can be assessed by a comparison of the therapeutic effects achieved in nonirradiated mice and in mice receiving a tolerated dose of total-body X radiation the day before tumor implantation, mice in which the immunological reactivity is depressed. The depression of the immunological response to the tumor by the drug can be assessed in some cases by an evaluation of the effects of the drug, administered before tumor implantation, on the curative action of an agent like 4,4′-diacetyl-diphenyl-urea-bis(guanylhydrazone), under conditions in which such action is known to depend on the immunological reaction of the host *(55, 56)*. Since some anticancer drugs do not seem to inhibit certain immunological responses when given before antigenic stimulation *(6, 7)*, it may not be always possible to show evidence of depression of the host response to L1210 following the experimental design outlined. For instance, methylglyoxal-bis(guanylhydrazone) (CH_3-G) inhibits transplantation immunity best when given after tumor grafting *(61)* and is active against L1210 by a similar schedule *(53)*. To evaluate the suppression of the immune reaction to the leukemia by this drug, one would have to give the drug in combination with an L1210 curative agent after tumor inoculation with a view to evaluation of the reduction in curative effects resulting from the action of CH_3-G on the defenses of the host. The data obtained in such experiments may be difficult to interpret, however, because of the conflicting antileukemic and immunosuppressive effects of a drug like CH_3-G.

A test system has been developed in this laboratory with the use of Sarcoma 180 in Swiss-Ha/ICR female mice fed a vitamin B_6-deficient diet for prolonged periods *(61)*. Under these conditions, the tumor regresses completely in more than 50% of the mice. These regressions depend on the immunological reaction of the host. In fact they are prevented by total-body irradiation *(54)*, neonatal thymectomy *(15)*, or immunological enhancement *(16)*. By giving a drug either during the week preceding tumor implantation or during the 1st or the 2d week following tumor implantation and by comparing results obtained in mice fed a complete or a vitamin B_6-deficient diet, in most cases one can assess the effects of the drug on tumor growth and those on early or ongoing transplantation immunity against Sarcoma 180 allografts *(59, 61)*. Systems based on the use of Sarcoma 180 may also be useful in the assessment of the relationships between drug effects on the one hand and the balance between immunological rejection and immunological enhancement on the other hand *(56)*.

It should be stressed that information obtained with the use of model systems based on tumor grafts cannot be directly extrapolated to situations involving autochthonous tumors. Caution in interpreting the potential validity of such information is in order not only when the data are obtained with the use of nonspecific tumor allografts, such as those of Sarcoma 180, or strain-specific tumor grafts, such as those of L1210, but also when they are obtained with the use of first or early transplant generations of autochthonous tumors. Nevertheless, such model systems can be used in an initial evaluation of the trigonal relationships among drugs, tumor grafts, and transplantation immunity, pending further validation of tentative conclusions in more rigorous systems based on the use of autochthonous tumors.

In addition to the development of tumor systems, it would seem potentially useful to evaluate the effects of anticancer drugs in systems based on immunological responses to antigens other than those associated with experimental tumors. Such test systems, as used in studies aimed at the development of effective immunosuppressive treatments of prospective clinical value, are not always very useful in cancer chemotherapy where the goal is to develop treatments capable of acting in cooperation with immunological reactions to tumors. Studies of the effects of anticancer drugs on the responses to normal grafts, such as skin or bone marrow, on graft-versus-host reactions, or on humoral antibodies must be geared to the questions relevant to cancer chemotherapy to fulfill their intended purposes. In general, as mentioned at the beginning of this section, the systems ought to be based on small antigenic differences and should measure effects on established, ongoing responses. In this laboratory, reciprocal skin grafts between DBA/2Ha-DD and DBA/2J mice are used, since these two sublines of the DBA/2 mouse are immunogenetically different but share the major H-2^d histocompatibility characteristics; thus these grafts are rejected slowly, in an average of about 30 days *(55)*. Studies of test systems based on bone marrow transplantation and graft-versus-host reactions *(73)* are quite relevant in view of the potential applications of bone marrow replacement and adoptive transfer of immune cells in attempts to combine chemotherapy with immunotherapy.

Model systems of particular interest permit an evaluation of the effects of drugs on the various cell types involved in delayed hypersensitivity reactions or in the hosts' responses to tumors. As mentioned in the preceding section of this discussion, in guinea pigs MTX selectively inhibited the formation of immunologically committed small lymphocytes without affecting the formation of immunoblasts in the cortex of lymph nodes draining an area of application of oxazolone *(86)*. Delayed hypersensitivity systems may be usefully developed in attempts to evaluate the possibility that some antitumor treatments block the response at certain specific stages of its development and that a reversal of such a blockage leads to an increased reaction of the host at the end of drug treatment. Test systems similar to those just mentioned might be developed to study the changes occurring in regional lymph nodes draining the site of a primary or implanted tumor. Indeed, as recent studies indicate *(28)*, such changes might be different from those occurring during a delayed hypersensitivity reaction to a compound such as oxazolone and might result from an effect of the tumor on the lymph node function. The cellular characteristics of the immunological response to primary tumors are relatively unknown in comparison with the progress being made in the identification of the cell types and interrelationship involved in antibody responses. When basic information is acquired on the cellular phenomena that are part of the host's response to tumors, then some of the questions raised in this discussion can be answered. Pharmacological studies on the action of anticancer agents may indeed contribute significantly to such a clarification.

Concluding Remarks

As the preceding discussion indicates, despite the limitations of the presently used anticancer drugs and of the types of immunotherapy that can be attempted and despite the intrinsic inefficiency of the immunological response to autochthonous tumors, hopefully successful treatments of neoplastic diseases may be achieved based on the cooperative effects of cancer chemotherapy and immunity. The antiproliferative and cytotoxic drugs now available may reduce the size of the tumor markedly, but are usually not selective enough to kill the last tumor cells. The immunological reactions to immunogenic tumors are intrinsically specific and therefore selective for the tumor, but are not efficient enough to affect significantly large neoplastic masses. Tumors may be reduced in size by drugs to the point of becoming a ready target of natural immunity or immunotherapy.

A substantial limitation in this combined approach to cancer therapeutics may be represented by the fact that most anticancer drugs can also inhibit immunological responses. Yet, a critical survey of the information now available suggests that the difficulties to be met in this respect are not as insurmountable as they may appear to be. In fact, as discussed earlier, in general, anticancer drugs are not very effective in reducing pre-existent or ongoing immunological responses and thus may not significantly impair established reactions of hosts to autochthonous tumors. Moreover, increasing amounts of evidence obtained in animals and in man support the view that it may be possible to exploit chemotherapy and immunity sequentially, despite the immunosuppression possibly occurring during drug treatment. In this respect, variations in drug regimens may significantly alter the degree of selective recovery of the immunological apparatus.

In view of the possibility that also in man, as is the case in some animal tumor systems, the growth of the neoplasm is influenced by the balance between immunological rejection and immunological enhancement, it may be desirable to study the possibility of exerting therapeutic effects by treatments selectively inhibiting humoral antibody responses.

It is generally known that a great deal of specificity is involved in the action of antitumor drugs on various types of immunological responses, specificity which in turn leads to a great deal of variability of effects, depending on the condition examined. This specificity is based not only on pharmacological parameters, most of which are species dependent, but also on immunological variables involving both the antigen and the mechanisms of the host's response. Because of this variability and because relatively little is known about the modalities of tumor immunity in man, at this time it is difficult to formulate any valid generalization in the consideration of the possible clinical interrelationships between cancer chemotherapy and immunity. A number of relevant preclinical test systems should be developed, however, to provide means of determining the optimal conditions of treatment with each drug or drug combinations that would allow the maximum exploitation of host defenses. Although the preclinical studies should provide basic knowledge on the selectivity of drug action and may lead to the formulation of conclusions applicable to clinical situations, obviously many of the questions raised in this discussion will be finally answered only when data become available on the interrelationships between the effects of antitumor agents and the response to tumors in the cancer patient, under the very conditions of clinical chemotherapy.

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Prognostic Significance of Cutaneous Anergy for the Surgical Treatment of Solid Neoplasms[1, 2]

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DURING the past decade, it has become increasingly evident that cancer cells have acquired new antigens during neoplastic transformation which are not found in their normal counterparts. Tumor-specific antigens have been demonstrated in almost every adequately studied virus-induced *(1)* or chemical carcinogen-induced neoplasm *(2)*, as well as in certain spontaneous tumors of laboratory animals *(3)*. The importance of the host immune response against these antigens is emphasized by the observations that procedures which impair this response, such as neonatal thymectomy, X irradiation, or the use of chemotherapeutic drugs or antilymphocyte serum, favor the emergence and progression of virus-induced and chemical carcinogen-induced tumors of laboratory animals.

It is logical to assume that human neoplasms contain cancer-specific antigens similar to those found in animal tumors, but until recently there was little evidence for the existence of such antigens. Since the tumor transplantation technique used to demonstrate tumor-specific antigens in animal neoplasms was not applicable to man, it was necessary to study these antigens by other methods. Recently, the application of sensitive serological techniques has revealed the presence of tumor-associated antigens in a variety of human neoplasms, including Burkitt's lymphoma *(4, 5)*, malignant melanoma *(6–8)*, neuroblastoma *(9)*, skeletal and soft-tissue sarcomas *(10, 11)*, colonic neoplasms *(12, 13)*, and other tumors *(13)*. Thus, there is increasing evidence that human cancers, like animal neoplasms, contain tumor-specific antigens which are immunogenic in the autologous host.

Clinical evidence demonstrating the importance of the patient's ability to react against these antigens comes from the congenital immunologic deficiency states, such as telangiectasia ataxia, where there is a high incidence of malignant disease *(14)*. Furthermore, the recent reports of the emergence of malignancies in patients on immunosuppressive treatment for renal homograft prolongation emphasizes the clinical importance of immunologic factors in malignancy *(15)*. Thus, there is increasing evidence not only that human malignancies do contain tumor-specific antigens, but also that the patient's immune response against these tumor antigens may be important in the development and progression of malignancy.

Previous investigations of immunologic capacities of cancer patients can be grouped into two categories: those concerned with humoral antibody production and those dealing with cell-mediated reactions.

Most investigators studying humoral antibody formation to known antigenic substances *(16–20)* have concluded that, in most patients with either solid or lymphoreticular neoplasms, the ability to form humoral antibodies to a variety of antigenic substances, even in the presence of advanced disease, is not impaired. Kelly *et al. (21)* and others *(22–27)* examined the cell-mediated reactions in patients with lymphoreticular neoplasia; they found a significant impairment of the ability of these patients to manifest delayed cutaneous hypersensitivity to a variety of common skin test antigens as well as prolongation of skin homografts. These results are not surprising, since these malignancies usually diffusely involved the immune effector system. However, patients with solid neoplasms were also found to have impaired delayed cutaneous hypersensitivity *(28)*, but many of these had far-advanced disease *(29–31)*. Subsequently, Solowey *et al. (32, 33)* reported decreased reactivity to a battery

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[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

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of skin test antigens in patients with more localized, malignant, solid tumors.

In the present report, we studied the capacity of preoperative patients with localized, solid neoplasms to display delayed cutaneous hypersensitivity to a variety of skin test antigens. In addition, we attempted to relate this immunologic reactivity to the subsequent course of the patients' malignant disease.

MATERIALS AND METHODS

Patients.—The patients ranged in age from 14–70 years. Patients older than 70 years of age were excluded because immunologic reactivity declines with advancing age *(34, 35)*. Also excluded were patients receiving chemotherapeutic drugs or steroids and those who had received X radiation within 4 months of the test. The patients included had neoplasms of the head and neck, skin, breast, colon, female genital tract, or sarcomas. Patients were tested before surgery when they were ambulatory, either in the clinic before admission or during their diagnostic evaluation. All were considered to have resectable neoplasms prior to surgery. Also included were 20 normal persons (hospital personnel and relatives of hospitalized patients, ages 18–65), patients with benign neoplasia, a group of cancer patients surviving more than 5 years after treatment, and a small group of patients with well-documented spontaneous regression of untreated, metastatic, malignant disease.

2,4-Dinitrochlorobenzene (DNCB)[4] sensitization and testing.—We used the technique previously described by Brown *et al. (36)*. This technique, which sensitizes over 95% of normal persons, is summarized as follows. The right arm and forearm were cleansed with acetone. Then a sensitizing dose of 2,000 μg of DNCB, dissolved in 0.1 ml of acetone (0.025 M) *(37)*, was applied topically to an area of normal skin on the volar aspect of the right arm, confined by a plastic ring of 2.2 cm in diameter. Doses of 100 and 50 μg were simultaneously applied to the ipsilateral right forearm to test for previous sensitization. The acetone was evaporated, and the test site was occluded with a dressing for 48 hours. The challenge doses of 100 and 50 μg were reapplied to the ipsilateral forearm after 14 days and occluded for 24 hours. The reactions were recorded at 48 and 72 hours. Reactions were positive if induration was present in an area of erythema covering at least one-half the test site.

No systemic reactions were found with this procedure. However, full-thickness skin loss was observed in one patient at the 100 μg challenge site which responded with complete re-epithelialization (within 10 days) to topical steroid cream. Some evidence of reaction—erythema or slightly increased pigmentation at the challenge site—usually remained for 2–3 weeks and occasionally up to 1 month after the test.

Skin test antigens.—A battery of 7 common skin test antigens were used, including blastomycin,[5] coccidioidin,[6] histoplasmin,[5] mumps skin test antigen,[7] mixed trees,[8] dermatophytin,[8] and purified protein derivative.[5] These were given as intradermal injections or as a scratch test to the volar skin of the forearm, as recommended by the manufacturer. No serious systemic or local reactions were observed. Readings were recorded at 24, 48, and 72 hours. They were graded positive if edema and induration were present in an area of erythema greater than 5 mm in diameter.

RESULTS

Incidence of Reactivity to DNCB

Table 1 shows that only 64% (70/110) of preoperative cancer patients had delayed cutaneous hypersensitivity to DNCB after controlled sensitization. This value was significantly different from that for our control group ($P < 0.005$ by χ^2) and for the control groups reported by others *(29, 35–37)*. Patients with benign disease, long-term survival following treatment of malignant neoplasms, or spontaneous regressions had normal delayed cutaneous hypersensitivity to DNCB. Ninety percent (63/70) of the operable patients free of disease 6 months after treatment were positive to DNCB. However, only 10% (7/70) of those patients who were either inoperable or who developed metastasis within 6 months after surgery reacted to DNCB preoperatively. This lower incidence of reactivity to DNCB exhibited by those patients who developed progressive disease was significantly different ($P < 0.005$ by χ^2) from the 90% reactivity exhibited by operable patients free of disease for 6 months after treatment. Seven patients with malignant melanoma or skeletal or soft-tissue sarcomas, who reacted to DNCB preoperatively, subsequently developed widespread metastasis and retained their ability to react to DNCB until their death. No patients negative preoperatively converted to positive after removal of all gross tumor.

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Table 1.—Incidence of delayed cutaneous hypersensitivity to DNCB and common skin test antigen in patients tested

	DNCB		Skin test antigens	
Type of Patients	Number positive / No. tested	% positive	Number positive to 1 or more / No. tested	% positive
Control	19/20*	95	18/20†	90
Benign	10/10	100	4/5	80
Long-term survival	14/14	100	6/10	60
Spontaneous regression	4/4	100	2/4	50
All patients preoperative	70/110*	64	30/76†	39
Free of disease (6 months)	63/70‡	90	26/47‡	55
Recurrence or inoperable	7/70‡	10	6/29‡	20

*Values significantly different—P by $\chi^2 < 0.005$.

†Values significantly different—P by $\chi^2 < 0.005$.

‡Values significantly different—P by $\chi^2 < 0.005$.

§Values significantly different—P by $\chi^2 < 0.01$.

Incidence of Reactivity to the Common Skin Test Antigens

The incidence of reactivity to 1 or more of the antigens of the preoperative group (39%—30/76) was significantly different from the 90% (18/20) of the control group ($P < 0.005$ by χ^2). Those patients with benign diseases (80%—4/5), long-term survivors (60%—6/10), and patients with spontaneous regression (50%—2/4) reacted to 1 or more of the antigens. Of those operable patients free of disease for 6 months, 55% (26/47) were positive preoperatively; whereas, of those who were inoperable or who developed early metastasis, only 20% (6/29) reacted to the antigens ($P < 0.01$ by χ^2). Again, no patients converted to positive upon rechallenge after removal of the gross tumor, but 4 patients who were positive to mumps lost their ability to display delayed cutaneous hypersensitivity as their disease became widespread. Two patients retained the reaction until they died.

Correlation Between Immunologic Reactivity and Results of Cancer Surgery

The capacity to react to DNCB was found to correlate closely with the results of cancer surgery (table 2). Of the patients who were DNCB positive, 90% (63/70) were found to be free of disease at 6 months; whereas, of those who were DNCB negative, 97% (39/40) were found to be inoperable or developed recurrence within 6 months. Twenty-one patients were considered inoperable because distant nodal metastasis was found at surgery, and six additional patients were considered inoperable because their previously local disease had extended.

It was found that DNCB reactivity more closely predicted the subsequent course of neoplastic disease in a given patient than the common antigens, since only 3% of patients free of disease were nonreactive to DNCB but 45% of these patients who were free of disease were negative to all common skin test antigens.

DISCUSSION

The results of this study indicate that patients who had delayed cutaneous hypersensitivity to DNCB and the common skin test antigens had a better short-term prognosis after definitive cancer surgery than those who were anergic.

The explanation for the presence of anergy is not known. It is clear that anergy is closely associated with metastatic disease which was the cause of inoperability in 21/27 patients and subsequently developed in 12/13 DNCB-negative patients that had undergone definitive surgery. Possibly, anergy preceded the development of

Table 2.—Correlation between immunologic reactivity and the results of cancer surgery

			Reactivity to common antigens	
Type of patients	DNCB positive	DNCB negative	Positive to 1 or more	All negative
Free of disease (6 months)	90% (63/70)*	3%† (1/40)	55%‡ (26/47)	45% (21/47)
Inoperable or recurrence	10% (7/70)*	97% (39/40)†	21%‡ (6/29)	79% (23/29)
a) Inoperable	4% (3/70)	100% (27/27)	21% (4/19)	79% (14/19)
b) Recurrence or metastasis	6% (4/70)	92% (12/13)	20% (2/10)	80% (8/10)

*Values significantly different—*P* by $\chi^2 < 0.005$.
†Values significantly different—*P* by $\chi^2 < 0.005$.
‡Values significantly different—*P* by $\chi^2 < 0.01$.

the metastatic disease and may have facilitated the early occurrence of widespread disease. However, metastases are not always associated with anergy, since 7 of our patients with melanomas, sarcomas, or widespread metastases retained the ability to react to DNCB. These observations and those of Krant *et al.* *(29)* indicate that cutaneous anergy must be caused by mechanisms other than the presence of metastatic disease *per se.*

The ability to display delayed cutaneous hypersensitivity to DNCB is more closely correlated with the subsequent course of the malignant processes than the reactions of the common skin test antigens. A possible explanation is that the elicitation of delayed cutaneous hypersensitivity requires adequate prior exposure to the specific antigen as well as the ability to manifest this recognition at the time of challenge. It is difficult to be certain whether a patient has had adequate exposure to the common skin test antigens. In contrast, controlled sensitization to DNCB is effective in over 95% of normal people. Furthermore, it tests the ability of the host to become sensitized to a newly encountered antigen and manifest this sensitivity at the time of challenge.

Whether anergy results from impairment of the afferent (sensitizing) or efferent (effector) immune mechanism is not known. Presumably, if most patients with malignant tumors could not react to the recently encountered DNCB but could react to the previously encountered common antigens, the defect would be in the afferent mechanism. Since most patients positive to the common antigens could also react to DNCB (48/50), no conclusion as to the site of the defect can be made from our data.

The observation that patients who are unable to develop delayed cutaneous hypersensitivity to DNCB have a high incidence of early recurrence following definitive cancer surgery may be useful in the management of patients on whom radical surgical procedures are contemplated. This appears to be true for patients with squamous cell malignancies of the head and neck or uterine cervix, which made up most of the patients studied. A larger number of patients in each category will be necessary to determine whether cutaneous anergy will follow a similar association with the progression of adenocarcinomas of the breast, gastrointestinal tract, melanomas, and skeletal and soft-tissue sarcomas.

Increasing evidence that human malignancies contain tumor-specific antigens provides a firm theoretical basis for observed relationships between immunologic competence and the progression of human neoplastic disease reported in this study. These observations also provide a more rational basis for future immunotherapy to control malignant disease. Hopefully, the evaluation of cell-mediated immunologic reactivity will be useful in planning this type of therapy, as active immunization in an immunologically competent host and passive immunotherapy in an anergic host would appear to have the greatest hope of success.

SUMMARY

One hundred fifty-eight patients were tested for their ability to develop delayed cutaneous hyper-

sensitivity to DNCB and to react to 7 common skin test antigens. After sensitization, over 95% of normal patients, but only 64% of patients with potentially resectable neoplasms, had delayed cutaneous hypersensitivity to DNCB. A correlation is suggested between the inability to react to DNCB and the incidence of either inoperability, local recurrence, or distant metastases within 6 months post surgery. Ninety-seven percent (39/40) of patients who failed to react to DNCB were inoperable or developed early recurrence, whereas 90% (63/70) of patients who reacted to DNCB were free of disease for 6 months; but many of these patients were nonreactive to all of the common skin test antigens. These studies suggest that there is a significant correlation between cell-mediated immunologic reactivity as measured by delayed cutaneous hypersensitivity to DNCB and the course of malignant disease after definitive cancer surgery.

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Cellular Immune Responses and Prognosis in Malignant Lymphomas[1, 2]

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IT IS well known that patients with Hodgkin's disease have impairment of cellular immune mechanisms, while antibody formation in these patients is normal or near-normal during most of the course of the disease. We have measured the delayed skin test responses of our patients to a number of common antigens for several years and have noted an apparent correlation between such responses and the clinical course of the patients. An example of this is shown in table 1. Even though long-term survivors were skin tested relatively late in the course of the disease, by which time considerable depression of cellular immune mechanisms would have been expected, they had a higher incidence of positive responses to tuberculin and mumps antigen than patients who died of Hodgkin's disease within 10 years.

Table 1.—Delayed skin test responses to second-strength purified protein derivative and mumps antigen in Hodgkin's disease, according to survival

Test results	22 long-term survivors* (over 10 years)	42 patients who died within 10 years
Positive to both antigens	7 (32%)	3 (7%)
Positive to one antigen	9 (41%)	13 (31%)
Negative to both antigens	6 (27%)	26 (62%)

*These patients were tested later in the course of their disease than were the short-term survivors.

The association between the status of cellular immune responses and the course of Hodgkin's disease, noted in retrospective reviews, was deemed sufficiently interesting to warrant prospective study. Accordingly, a fairly large group of patients with Hodgkin's disease and smaller groups with other malignant lymphomas were identified a few years ago for correlation of immunologic status with the subsequent clinical course. Almost all patients seen in our outpatient clinic, or admitted to our inpatient service, during a period of 18 months, were registered for such follow-up. Physicians not participating in this immunologic study classified patients according to sex, histologic diagnosis, stage, and activity of disease. A coinvestigator subsequently reviewed the patients' charts to verify the accuracy of these classifications; he had no knowledge of the results of the immunologic tests, which were kept in a special file. The patients selected for prospective study included a broad spectrum of those with early and late malignant lymphoma. In general, terminal patients and patients whose course was changing rapidly were excluded. Thus, although many patients selected were on maintenance therapeutic routines, and some had active disease about to receive energetic chemotherapy or irradiation, most of them were individuals with stable and satisfactorily controlled disease, whose prognosis at the time of selection appeared relatively favorable. Certain subgroups from this population of patients were selected for special studies and form the basis for the present report.

Cellular immune mechanisms are rather uniformly depressed during active, symptomatic malignant lymphoma *(1)* and during steroid administration *(2)*. Thus negative skin tests during such periods have relatively little significance. A generation or two ago, when almost all adults were tuberculin positive, anergy to tuberculin could be considered *prima facie* evidence of immunologic deficiency. This is no longer true, and it is usually impossible to determine whether a given individual has been exposed to the tubercle bacillus. Thus the tuberculin test has lost much of its value as a measure of cellular immune responsiveness. However, the mumps skin test is quite useful, since almost all adults will have had overt or unrecognized mumps infection, and approximately 85% of the normal population will have positive mumps skin test reactions.

The data presented below are based on review of the records of patients identified for prospective study on specific dates, when 1) their malignant

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[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

lymphoma was quiescent or only slightly active and not producing functional impairment, 2) they were not receiving corticosteroid therapy, and 3) skin test responses to second-strength purified protein derivative (PPD) were negative.

Table 2 presents the skin test responses of these patients to mumps antigen, according to stage of disease. Over half the patients with localized or regional Hodgkin's disease and three-fourths of such patients with lymphosarcoma or reticulum cell sarcoma had positive mumps skin tests. In contrast, only one-fifth the patients with disseminated disease reacted to mumps antigen. This finding confirms previous reports from our group and others that immunologic deficiency, though often demonstrable quite early in malignant lymphoma, is much more striking in the later, disseminated stages.

TABLE 2.—Frequency of positive mumps skin tests among tuberculin-negative patients with malignant lymphoma, according to stage

Category	Number of patients	Percent with positive mumps tests
Hodgkin's disease		
Stages I and II	46	57
Stages III and IV	54	20
Lymphosarcoma		
Stages I and II	11	73
Stages III and IV	32	28
Reticulum cell sarcoma		
Stages I and II	8	88
Stages III and IV	32	16

Only in Hodgkin's disease did we have a sufficient number of mumps-negative patients, in Stages I and II, to look for a correlation of the mumps response with fate. However, only 10% of the patients with Stage I or II Hodgkin's disease have died during 3–4 years of follow-up. Thus no evaluation of this relationship is yet possible.

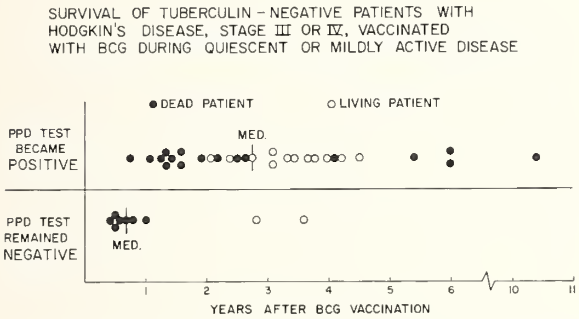
In disseminated lymphoma, the mumps skin test appeared to have some significance (table 3). Although the reliability of this conclusion is prejudiced by the few mumps-positive patients in each group, there seems to be a correlation between the finding of a positive skin test response and subsequent survival. Most mumps-negative patients in each diagnostic category died of their disease, whereas 75% of all the mumps-positive patients were alive at 3 years.

TABLE 3.—Survival, from date of mumps skin test, of tuberculin-negative patients with disseminated malignant lymphoma (Stages III and IV)

Category	Number of patients	Percent of patients at risk, alive at: 1 year	2 years	3 years
Hodgkin's disease				
Mumps-positive	11	91	73	60
Mumps-negative	43	83	65	46
Lymphosarcoma				
Mumps-positive	9	89	78	75
Mumps-negative	23	87	52	35
Reticulum cell sarcoma				
Mumps-positive	5	100	100	100
Mumps-negative	27	78	59	42

To overcome the problems associated with reliance on the mumps skin test response, whose intensity correlates inversely with age and which is lost in most patients with disseminated disease, we administered a standardized immunologic challenge to a group of patients with disseminated disease. Bacillus Calmette-Guérin (BCG) vaccination was selected because it constitutes a suitably strong immunologic stimulus, is easy to administer, with little hazard to patient or physician, has no side effects, and is potentially beneficial to these patients, who are known to have increased susceptibility to tuberculosis. Patients were inoculated with the doses of BCG vaccine used routinely in tuberculosis-control programs; they were retested with PPD at least once, and usually twice, during the next 16 weeks. Since active disease vitiates interpretation of negative skin test responses *(1)*, patients who developed progressive and uncontrolled disease after BCG vaccination were excluded from analysis of survival. Thus survival with well-controlled disease for at least 2–4 months after BCG vaccination was ensured for all patients included in the final evaluation.

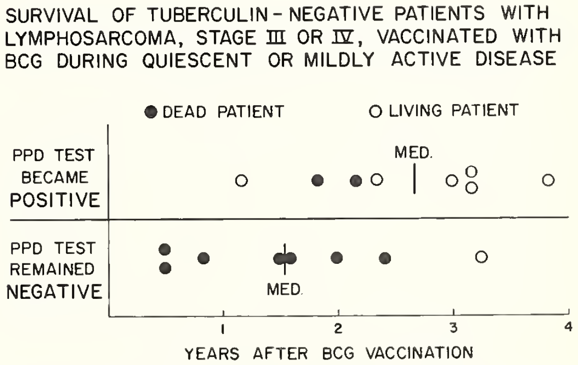
Text-figure 1 presents the current status of 38 patients with Hodgkin's disease in the BCG study. Thirty-two of these patients have been reported on previously *(3)*. Of these 38 patients, 9 failed to convert their tuberculin response; 7 of these died within 12 months of BCG vaccination. In contrast, only 1 of the 29 patients who developed at least a transiently positive reaction to PPD died within a year of BCG vaccination, and the median survival of this group, currently 33 months, is still increasing. The survival difference between these 2 groups of patients is highly significant (< 0.01) and indicates a distinct correlation between cellular immune capacity and prognosis. Although there was also some correlation between survival after vaccination and other characteristics (sex, age, prior duration of disease), this was not nearly as striking as that with skin test conversion. Tuberculin conversion was more common among females than among males and less common among elderly patients. These findings suggest that the somewhat better survival of women with Hodgkin's disease may be explained by their greater immunologic capacity, and that the unfavorable prognostic import of advanced age may be related to the loss of immune responsiveness. There were no significant correlations between stage of disease, or individual disease manifestations, and survival in this group of patients. Prior duration of disease was *longer* among the converted patients than among those who remained tuberculin-negative.


SURVIVAL OF TUBERCULIN - NEGATIVE PATIENTS WITH HODGKIN'S DISEASE, STAGE III OR IV, VACCINATED WITH BCG DURING QUIESCENT OR MILDLY ACTIVE DISEASE

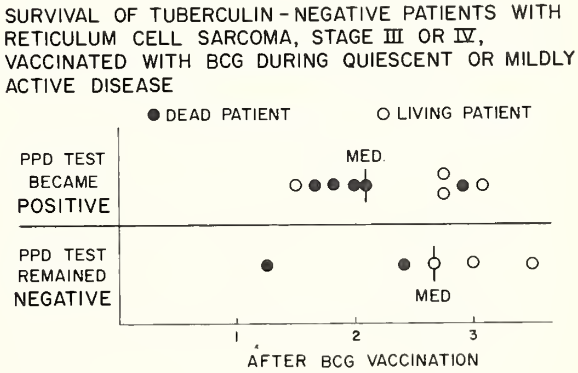
Text-figure 1.—Survival of patients with disseminated Hodgkin's disease, according to tuberculin response after BCG vaccination.

Most converted patients reacted only to second-strength PPD, but many also reacted to intermediate PPD. Some of these patients, previously mumps-negative, developed positive skin tests to mumps antigen after BCG vaccination. Thus BCG infection has some nonspecific stimulatory effect on cellular immune responses, in addition to specifically inducing sensitization to tuberculin; this is consistent with studies of BCG action in experimental animals.

A small group of patients with disseminated lymphosarcoma was included in the BCG study (text-fig. 2). Although the difference in survival between the converted patients and those who remained anergic to second-strength PPD was not nearly as impressive as that in Hodgkin's disease, the pattern was generally similar. On the other hand, among 14 patients with reticulum cell sarcoma, there was no correlation between tuberculin conversion and survival (text-fig. 3). Whether this reflects a fundamental difference between reticulum cell sarcoma and the other malignant lymphomas, or is attributable simply to individual differences among the small numbers of patients in each group, is not clear. Interpretation of these results will require detailed review of the records of these patients, which has not yet been accomplished. Obviously, study of a much larger group will be necessary to determine the value of immunologic testing in reticulum cell sarcoma.


SURVIVAL OF TUBERCULIN - NEGATIVE PATIENTS WITH LYMPHOSARCOMA, STAGE III OR IV, VACCINATED WITH BCG DURING QUIESCENT OR MILDLY ACTIVE DISEASE

Text-figure 2.—Survival of patients with disseminated lymphosarcoma, according to tuberculin response after BCG vaccination.


SURVIVAL OF TUBERCULIN - NEGATIVE PATIENTS WITH RETICULUM CELL SARCOMA, STAGE III OR IV, VACCINATED WITH BCG DURING QUIESCENT OR MILDLY ACTIVE DISEASE

Text-figure 3.—Survival of patients with disseminated reticulum cell sarcoma, according to tuberculin response after BCG vaccination.

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DISCUSSION

Dr. Krivit: Can you relate this immunologic work to white blood cell counts (wbc) or lymphocyte counts? What is the relationship, and what type of quantitation have you done?

Dr. Morton: Although we have not analyzed these other factors in detail, we have looked at them superficially. We have not found very impressive correlations with wbc and differential. This lack of correlation is clouded in part by the fact that some patients were on maintenance chemotherapy during the study. Chemotherapy did not exclude these patients from the study, provided it did not include prednisone or massive combinations, but there were patients on vinblastine and chlorambucil which would, of course, affect the wbc. We also have not done a careful point-to-point correlation with histologic type—another interesting factor to study. But the results of immunologic testing in patients with Hodgkin's disease are strikingly significant.

Dr. Nathanson: Dr. Morton, you stated that lack of immunologic reactivity in patients with solid tumors who did poorly was presumably related to their subsequently poor prognosis. It is not clear to me why the reverse interpretation could not just as well have been made. Obviously, patients who do poorly are going to be those harboring disease when you study them, those who have not been cured, or (the opposite) those who presumably are, in the same proportion at least, cured. Therefore, the disease can cause the immunologic paralysis, rather than immunologic paralysis causing the disease.

Dr. Morton: Perhaps in the group classified inoperable or in the local recurrence group, we did have 20 patients whose disease was eradicated locally by the same sort of procedure used in the 2,4-dinitrochlorobenzene (DNCB)-positive group; there was no gross disease. The difference is that the disease returned locally within 6 months in one group.

Dr. Nathanson: Obviously that implies the disease was microscopic, not gross.

Dr. Morton: Are you suggesting that the patients were selected on the basis of the DNCB test, and it just so happened that all of those patients who were DNCB negative had residual microscopic disease and those who were DNCB positive had no residual microscopic disease?

All of these patients were preoperative. They all presented with malignant disease. They all went through an intensive preoperative evaluation, including chest X rays, tomography, upper gastrointestinal tract tests, barium enema, IVP, and cystoscopy. We do have a group of about 25 or 30 patients in whom the challenge skin test was not applied until after surgery. I have not included these patients. Only those prospective, preoperative patients were discussed.

Dr. Sokal: The question is a very serious one and one that has concerned me, and I think it applies also to Dr. Morton. Basically, at issue here is: Which is the chicken and which is the egg? Are the patients immunologically deficient because their tumor has reached a stage where it is going to kill them soon, or does the tumor kill them sooner because they are immunologically deficient? At this time we cannot really give a definite answer to this question. From the standpoint of the topic of this Conference, mainly the use of testing to predict response, it is irrelevant because both Dr. Morton's and my data for Hodgkin's disease show very good predictive values. But the more important and fundamental point is doing something active rather than just selecting patients who are going to do better. I agree with Dr. Morton that these studies show, at least in part, that the patient with better immunologic capacity is using it to control his disease. But I think that, in part, there is no question that these studies imply the disease has gone to the point where it depresses immunologic response.

Dr. Hall: When you look at patients with early Hodgkin's disease of different histologic types, patients with nodular sclerosis, and patients with lymphocyte predominance, the patients with nodular sclerosis tend to have a worse prognosis but nonetheless their tumors are of the same anatomic stage. If what you say is true, then we might be able, within each restricted group, to define groups with better and worse prognosis.

Dr. Regelson: This approach would still be indirect and would lead to some difficulty. The only way to answer the question is in an active sense: to prove that, by manipulating the patient's immunity, you can change his prognosis. This has already been done, unintentionally, in transplant patients who have been intensively immunosuppressed for long periods of time. In these patients, quite clearly tumors that should not have grown, grew and could have killed the patients. What we must do now is the reverse: to show that, by improving immunologic activity in patients, you increase patients' survival. In my Department, these studies have been recently initiated.

Dr. Mihich: In terms of the prediction of survival or therapeutic effectiveness, the response to bacillus Calmette Guérin (BCG) or to the antigens that were tested, and the actual observation of survival, the test systems are valid. From the viewpoint of the fundamental question of trying to manipulate the host and to exploit whatever imbalance in the direction of cellular defenses one might elicit from the therapeutic point of view, the stimulation of a patient or an animal with nontumor antigens is not very accurate. All we know of experimental immunology suggests that the response and the suppression of the response by drugs or by treatment are highly specific in relation to the type, the dose, or the form of presentation of the antigen. While the predictability aspect can be perhaps assessed in this fashion, therapeutic utilization in the host cannot. Now we are near the point where we can utilize *in vitro* systems to assess the reactivity of the lymphoid cell or circulating leukocyte from a patient against his own tumor.

Dr. Regelson: Dr. Mihich made one mistake when he discounted all nonspecific immunization. It has been repeatedly demonstrated in animal systems that BCG vaccination does confer resistance to tumors; I am sure this resistance is nonspecific.

Dr. Nathanson: The following point is perhaps valid: If the patient cannot manifest this immune response, to such a potent immunogenic response, to DNCB, tuberculin, or BCG, then it cannot be expected that he will manifest immune response to the tumor-specific antigens. We recently reported immunotherapy in a small series of patients with melanoma. We found a one-to-one correlation between the ability of the patients to be sensitized to DNCB, to convert from a negative to positive tuberculin, and to manifest a fourfold increase to an antibody-specific form of malignant melanoma. In other words, there was a correlation between the ability of the patients to manifest an immune response to these "nonspecific stimulants" and the ability of the patients to manifest a specific immune response to tumor antigen associated with a malignant melanoma. This also correlated with the therapeutic response.

Dr. Prager: We have studied a highly sensitive lymphoma. Although we could not demonstrate an immunological response in the animal without therapeutic intervention, if we treated the tumor-bearing animal, a very potent and long-lived immunity resulted. Have you attempted to use a more specific test like a colony inhibition?

Dr. Morton: This test is very difficult. It is really a nonquantitative test. It is hard to compare the results obtained with a freshly explanted tumor today with the results you might obtain a month or more from now, because the tumors change in culture and even from time to time during the test. We have data on the serological response to tumors with specific antigens, malignant melanomas, and sarcomas. In sarcomas, patients with higher titers, *i.e.,* 1–128 and 1–256, do better with their disease than those with low titers, *i.e.,* 1–64. Furthermore, the titer usually rises after surgery, drops with recurrence of the tumor, and progressively drops to 0 with progression of the disease.

Dr. Heidelberger: Is it possible to do repeated DNCB challenges or is this a one-shot thing? I have heard that most antitumor drugs are immunosuppressive only in either the primary response or the secondary response. Is this so and, if so, would this not mitigate against the argument which has been used against treating with immunosuppressive drugs in chemotherapy?

Dr. Morton: With DNCB, you can challenge repeatedly, you can test for sensitivity at various periods, and most patients retain their sensitivity. Of the patients that we have followed to death, about 50% who are DNCB positive develop a recurrence from which they die.

Dr. Heidelberger: If you can do this repeatedly, then presumably you can follow not only the effect on a patient who was subsequently treated by chemotherapy, but you can study the effect of chemotherapy on the tumor and also study, perhaps separately, the effects of the chemotherapy on the immune response of the patient under conditions of treatment of his tumor.

Dr. Morton: I am not at all clear that we really know what is going on with these chemotherapeutic agents. Yes, these agents are immunosuppressive. But Schwartz has shown that, if you give immunosuppressive drugs in certain schedules and give your antigen at the right time, you can obtain an enhanced immune response. The whole effectiveness of chemotherapy may depend on the ability of the chemotherapeutic agent to reorder the disordered immune response.

Dr. Mihich: Regarding the second question of Dr. Heidelberger, the evidence available really supports both concepts, because most anticancer drugs are probably immunosuppressive when used at high doses during the early period after antigenic stimulation. In particular systems, not only the secondary response can be inhibited but also an on-going response can be inhibited to repeated stimulation with small doses of weak antigens, which would be the case in human tumors. Also, you have the clinical application of these drugs for the treatment of autoimmune disease, in which you certainly have an established immunologic response to a relatively weak antigen, coming in this case from normal tissue. Therefore, one cannot exclude the possibility that, with a continuing weak antigenic stimulation by an autochthonous tumor in man, these drugs might depress immunity to the same tumor, even though the immunity was established. The clinical information available from Hirsch and others indicates that the depression of the immunologic response by drugs is usually very short lasting and there is a complete recovery. For instance, the primary response is completely depressed within 72 hours after the end of an intensive combination of chemotherapy treatment of leukemia patients. With regard to the question of enhancing immunity by drugs which results in a chemotherapeutic effect, this is possible. This is why I mentioned that one might design a drug to alter the balance in favor of cellular defenses. Colony inhibition is one of the *in vitro* tests which might become useful in assessing the real activity of the lymphoid cells of a patient to his own tumor. From a therapeutic point of view, this is important. From a prognostic point of view, antigens are quite valid, as Dr. Heidelberger indicated.

Dr. Olson: Dr. Sokal, what is your explanation for the development of these types of cancer in Hodgkin's disease patients surviving a long time without treatment? Supposedly, these patients did not lose their immunological capacities and yet they did become accessible to the oncogenic effects of X rays.

Dr. Sokal: You are referring to leukemias in Hodgkin's disease which are presumably radiation induced. There is more to destroying the tumor than just being able to react to a strong stimulus such as BCG or DNCB, and there is more to becoming susceptible to tumor cell growth than we have yet identified. One thing that keeps puzzling me is the patient with breast carcinoma who is completely free of the disease for 15 years and then develops metastases all over. We immunologists like to think that the reason she was free of disease for 15 years was that her immune mechanisms controlled the residual cells. Then something happened and the residual cells suddenly began to grow like mad. Was it an immunosuppressant virus infection that she caught? We know that measles will wipe out cellular immune responses. I suspect that there are all kinds of minor viral illnesses that will wipe out tumor-specific cellular immune responses. I suggest that, in the course of daily existence, all kinds of things happen that we do not understand that might have caused these 3 patients to have developed leukemia. We irradiated many patients. At some crucial point in time, could these patients have come into contact with some virus causing a minor illness that happened to hit their immune response at the wrong time? I do not know.

Dr. Berlin: You began testing individuals who were BCG negative, presumably during an active phase of their disease. What is the probability that your studies were biased because you segregated out a group of patients, whose histories you did not know, who could have been BCG positive at one time but at the time of the studies were truly anergic? These would be the individuals with the poor prognosis by whatever immunologic terms you have.

Dr. Morton: Anyway you look at my series of patients, there is bias one way or another. You have pointed out one obvious possible bias: We may have included "losers" because these patients had been exposed to tuberculosis and had had positive responses which then became negative. In the other direction, the physicians who selected these patients were instructed to give us patients whose course could be guaranteed to be stable for a few months. So that a lot of losers were eliminated that way. We thus ended up with a group of patients who presumably did not ideally represent the total population, but the results were valid from the standpoint of consistency within the study.

Active and inactive diseases are classified in the following way. We have a rating scale of 0 for a patient who is totally asymptomatic, has a fully active disease, and who has no enlarging tumor mass. He can have a stable node, but no enlarging tumor mass, no anemia, etc. The scale goes up to 4+ for a patient who is on the critical list. The patients designated as "quiescent" for the purposes of this study have 0 and 1+ activity. Patients with 1+ activity are those who have minor disease manifestations, like slight anemia, or who have no manifestations but who have very slowly progressive disease, *i.e.,* enlarging nodes or mediastinal shadows, and that sort, but the patients classified 1+ must have a fully active disease. The main difficulty in segregating patients is to decide who has 1+ activity and who has 2+. There is a certain amount of individual judgment here, but the whole series was reviewed independently by a single individual. It turned out that, of about 30 patients, there were only about 5 or 10% possible classification differences by different observers. So it is reasonably valid.

Dr. Sokal: Probably most people who study Hodgkin's disease would question the indicators you use: complete remissions and the incomplete remissions. Anemia puts a patient in class B which generally carries with it a more severe prognosis. When these data become available, if they are not already available in the literature, we would like to see the classification of disease and the classification of activity conform to what we have agreed to in the Rye Classifications.

Dr. Morton: These data were published in *Cancer,* July or August of last year. The scheme that we set up is independent of the international staging and the A and B classification. I believe activity has nothing to do with staging; you can be stage 1 and have 2+ activity and so on.

Dr. Hall: I would not agree with the assumption that regression of the patient's tumor that was transplanted has anything to do with the fact that this was a tumor or has anything to do with tumor immunology. Presumably, the tumor grew as part of a histoincompatible system, along with the kidney. There are precious little data to support the view that immunosuppressive chemotherapy in man has ever demonstrably resulted in deterioration of the patient's status or increase in the growth of the tumor. I would like to ask a question of Dr. Morton that relates to the phraseology he used in describing his patients. Dr. Morton always included two groups of his patients in one group: inoperable and recurrent. This I find interesting because an inoperable situation is a biological condition very different from a recurrent situation. An inoperable patient might have already been suffering from many of the well-known widespread biological effects of even the smallest tumor—little tumors that cause hypercalcemia or hypertrophic osteoarthropathy. The whole thing may be reversed: The smallest tumor often has massive effects on the host. If you had in your two groups patients who had metabolic defects because they were in an inoperable condition as well as patients in fairly good shape yet who were going to have a recurrence later, you could confuse the two.

Dr. Morton: As a surgeon, I am not very concerned with the metabolic aspects. These are all patients that went to surgery and were explored. Now, most of the people that were later classified as inoperable had squamous cell carcinoma of the cervix which, by the routine preoperative evaluation, fell into the category of operable and were therefore explored. Their inoperability was usually due to the presence of periaortic lymph node metastases; there were 20 patients in that group. That is one-half of the inoperable or recurrent group; the other 20 patients were resectable and there was also recurrence in 6 months. Your point is well taken that we should break those two groups down. If we redefine the data by your criteria, these are all patients that were worked up completely and they all went to surgery and were explored. In that group were 20 patients who were DNCB negative and 20 patients who were inoperable because of metastases found at the time of surgery. Another 20 patients had either squamous carcinoma of the cervix or squamous cell carcinoma of the head and neck or melanomas that were DNCB negative and were resected, or they had the standard surgical treatment for the disease. As far as one could tell, they were equally cured surgically wise, marginwise, and so forth, and were as equally free from disease as those that were DNCB positive. Of 20, 19 developed a recurrence within 6 months. I agree that there are metabolic and other effects of tumors, but, in terms of looking at the patient grossly, there was no difference in those patients who were DNCB positive and those who were DNCB negative in terms of the amount of cancer removed or the extent of tumor. Furthermore, if it was just a matter of the size of the tumor, you would expect that the sarcoma patients who have metastasis should be DNCB negative because they have a lot of tumor, but they are DNCB positive. Furthermore you would expect that, as the DNCB-positive patient develops a recurrence and goes progressively downhill with growth of the tumor he would become DNCB negative. In fact, about half of the patients do and the other half do not. As far as I can see, there was no selection of patients. The data were recorded before the patient went to the operating room, and I do not think that they are biased in this way.

Dr. Mihich: However, in reply to your comment, Dr. Hall, I think that, even though we probably do not have clear evidence of a deterioration of the tumor growth during chemotherapy, we do have repeated evidence of infections, which are an index of a decreased immunological activity in certain patients with certain types of cancer during chemotherapy. Also, if the evidence being reported holds up, we will have to conclude that, in heavily immunosuppressed patients with transplanted kidneys, there is an increased incidence of reticulum cell sarcomas. This still would not hamper my faith in a cooperative action between chemotherapy and immunity, because those patients are heavily immunosuppressed with doses and regimens we would not use in cancer chemotherapy.

PANEL IV

GENETIC FACTORS

In Search of a Key[1, 2, 3]

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COMPLETE understanding of the biochemical basis of a drug-induced oncolytic lesion in human neoplastic disease should permit the selection of patients for treatment with the agent. This knowledge would also provide a molecular basis for: 1) testing the possibility of reinforcement or accentuation of drug action by combination therapy and 2) duplicating the same lesion by agents acting at different loci. Combination of these two approaches might create the metabolic pattern of drug response in neoplastic cells that otherwise would be insensitive to the drug.

The oncolytic lesion should be definable by relating cellular metabolism to drug response. The initial metabolic pattern of the neoplastic cell should be known, and the drug-induced changes observed. Serial sampling of the neoplastic cell population after drug administration to the patient would permit drug action to be mapped on selected parameters and should also reflect on the parameter any variation in drug metabolism, distribution, or excretion by patients.

White blood cells from patients with leukemia are a source of neoplastic cells that may be sampled serially with minimal inconvenience or discomfort to the patient. Alteration of the metabolic pattern in leukemic blood cells in serially obtained specimens over a 24-hour period or longer, may also indicate changes in the population of cells, in addition to specific changes by intoxicated cells. Selective destruction or shifts in the growth phase of specific populations of leukemic cells could give the appearance of altered metabolic patterns in drug-sensitive cells. Information of this type should prove useful in planning sequential drug therapy. Correlations between any of the observed changes and clinical response would provide a basis for selecting pharmacologically interesting parameters for further study.

The present necessity to depend on clinical criteria of response can be pharmacologically misleading and may result in the selection of only a fraction of the patients with drug-sensitive neoplasia. Variation in the disease status of patients, sensitivity of normal cells to drugs, and nutritional status contribute to the patient's tolerance of an oncolytic agent and potentiality for recovering a normal, or near-normal, hematologic pattern. Quantitative measurement of the contribution of these factors to response of the patient is unavailable for pharmacologic evaluation.

To compensate for the biological variation of patients with the same diagnosis, but pharmacologically different disease, advantage may be taken of the distinct differences in drug sensitivities observed for patients with acute lymphocytic leukemia, acute myelogenous leukemia (AML), or chronic myelogenous leukemia (CML). Of course, the variation in drug sensitivity of these various leukemias may be inherent in the nature of the total disease and not necessarily in the neoplastic cell. But the expanded approach provides needed information on the response of human neoplasia.

In the overall approach followed, the initial clinical studies started with extrapolations from preclinical, pharmacologic observations and returned to these preclinical systems with questions arising from the patient. Of all the available oncolytic agents, methotrexate (MTX) appeared, at the time we[4] started our studies, to have the fewest possible alternative mechanisms of action. Our working hypothesis was: MTX inhibition of dihydrofolate reductase initiated drug action *(16, 28, 46)*. We assumed that response was determined by the cellular requirement for tetrahydrofolate, the product of the dihydrofolate reductase-catalyzed reaction *(14)*. Variation in sensitivity to MTX

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[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

[3] Supported by Public Health Service research grants CA 11148 and CA 08480 and Public Health Service contract PH 43-63-169, all from the National Cancer Institute.

[4] The helpful collaboration contributed by Dr. Thomas C. Hall to much of the work which will be presented is gratefully acknowledged.

would presumably depend on a few pharmacologic relationships, *i.e.,* drug uptake, rate of recovery of enzyme activity, time required to kill a sensitive cell, capacity of the cell to circumvent the metabolic block imposed by the inhibition of dihydrofolate reductase, and possibly on unknown factors. Activation of MTX is not known to be required, and inactivation by humans probably does not occur to a significant degree *(23)*.

Support for the hypothesis that inhibition of dihydrofolate reductase activity was the basis for MTX action came from reports on the extreme sensitivity of this enzyme to MTX inhibition *(45, 46)* and the association of elevated levels of dihydrofolate reductase activity with resistance in experimental systems *(1, 13, 15, 18)*.

LACK OF CORRELATION BETWEEN DIHYDROFOLATE REDUCTASE AND RESPONSE TO MTX

Levels of Enzyme Activity

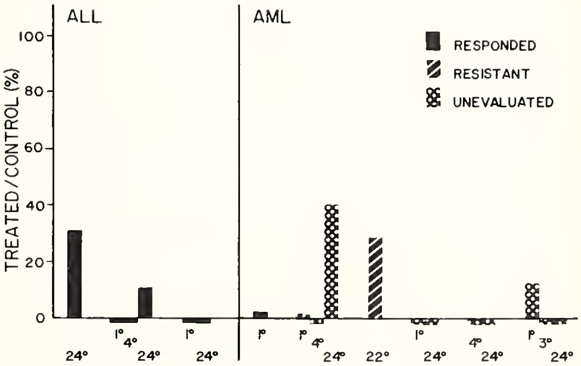
Dihydrofolate reductase catalyzes the formation of tetrahydrofolate with either folate or dihydrofolate as substrate. Measurement of the low level of dihydrofolate reductase activity in human cells required the development of an assay with isotopic folate as substrate *(20, 32)*. We needed an assay with folate because of the greater affinity of MTX for dihydrofolate reductase at *p*H 6, which was used with this substrate *(2)*. Although the drug-enzyme complex does dissociate *(44, 45)*, competition between folate and MTX cannot be demonstrated in the assay, and dissociation of the MTX-dihydrofolate reductase complex is considered negligible during the isolation of white blood cells and preparation of the homogenate.

The methodology required quantities of human cells that could not be readily obtained by marrow aspiration, and our intention to study changes in biochemical parameters following drug administration resulted in the decision to work with more easily obtainable blood cells. In support of this decision, there were only slight differences for the incorporation of deoxyuridine and thymidine into DNA by normal marrow and by marrow and peripheral leukocytes from patients with acute granulocytic leukemia *(35)*.

Bertino, Gabrio, and Huennekens encountered the problem of low levels of enzyme activity in human leukocytes, but they observed that dihydrofolate reductase activity in the peripheral blast cells of acute leukemia and in the immature cells of (CML) was elevated above the level in normal leukocytes *(6)*. Neither they nor we have observed significant differences in dihydrofolate reductase activity of white blood cells from patients with these leukemias *(7, 35)*. However, acute lymphocytic leukemia of children is much more responsive to MTX than either acute or chronic myelogenous leukemia of adults. Therefore, the variation in response to MTX of patients with these leukemias was not related to the pretreatment levels of enzyme activity. Moreover, resistance to MTX could not be correlated with elevated levels of dihydrofolate reductase activity in leukocytes from patients previously treated with MTX *(35)*.

MTX Inhibition of Dihydrofolate Reductase in Human Leukocytes

MTX inhibition of dihydrofolate reductase activity in peripheral leukocytes was measured with cells from leukemic patients. Pharmacologic doses of MTX were administered intravenously to the patients. Blood specimens were drawn before and at various times after drug administration. Dihydrofolate reductase activity of the isolated white blood cells was inhibited equally as effectively for the three different types of leukemia, and the recovery of enzyme activity was in the same range after 24 hours [*(33, 35)*; *see* text-fig. 1].



Text-figure 1.—At various intervals after MTX administration, dihydrofolate reductase activity of leukocyte homogenates was assayed and compared with the pretreatment level of activity. The methodology has been previously described *(37, 38)*. ALL = acute lymphocytic leukemia; AML = acute myelogenous leukemia.

The reports by Bertino and his associates *(3–5)* of increases in the levels of enzyme activity 1 week after MTX administration were confirmed with normal leukocytes from patients with nonhematologic malignant disease and with leukocytes from patients with CML, but not with leukocyte preparations from patients with acute leukemia *(33–35)*. To study the effect of the elevation of dihydrofolate reductase on the sensitivity of the enzyme activity to MTX, a small group of patients with CML were treated with MTX. One week later, a second pharmacologic dose of MTX was administered. The elevated dihydrofolate reductase activity was severely or completely inhibited by MTX. The recovery of enzyme activity 24 hours after the second dose of MTX was in the range observed for patients with acute lymphocytic leukemia. Therefore, we concluded that the drug-induced elevation observed in the white blood cells was not the basis for the insensitivity of CML to MTX. The drug-induced elevation of enzyme activity did not exceed the level observed in some patients with untreated leukemia.

The 4- to 7-day lag, before the drug-induced elevation of enzyme activity in blood leukocytes, has been suggested by Bertino and associates to indicate a response by the marrow *(3)*. Therefore, our attempt to correlate drug sensitivity with changes in the blood cells should be extended to a study of bone marrow.

Dihydrofolate Reductase Activity of Mouse Leukemias

Before deemphasizing dihydrofolate reductase activity as the prime determinant of sensitivity to MTX, several other studies were completed. Dihydrofolate reductase activity of a spectrum of transplantable mouse leukemias was assayed *(40)*. This group of mouse leukemias (generously provided by Dr. Isodore Wodinsky, together with the information on MTX sensitivity) varied naturally in their sensitivity to MTX. No correlation was observed between the effectiveness of MTX in prolonging the life of the tumor-bearing mouse and the level of dihydrofolate reductase activity. This observation concurred with the preceding observations for human leukocytes; it indicates that the *natural* variation in dihydrofolate reductase activity in leukemic cells is not the determinant of sensitivity to MTX.

Drug Uptake and Inhibition of Dihydrofolate Reductase

This spectrum of ascites cell tumors was also used in a collaborative study with Dr. Kessel *(25)*. *In vitro* uptake of MTX by these ascites tumor cells correlated with the effectiveness of MTX in prolonging the life of the host; *i.e.,* the greater the uptake of the drug by the ascites tumor the more effective the drug in prolonging the life of the host.

This variation in drug uptake did not prevent an inhibition of dihydrofolate reductase in ascites tumor cells of L1210, L1210/MTX, or P329 when the drug was administered intraperitoneally *(39)*. The rate of MTX uptake by L1210/MTX was approximately one-fifth as rapid as that for L1210. P329, a naturally MTX-insensitive cell line, was slightly less than one-half as efficient as L1210 in the uptake of MTX at a concentration of 0.1 μg of drug/ml.

Recovery of enzyme activity after MTX administration was much more rapid in L1210/MTX than in L1210 or, the drug-insensitive cell line, P329. Although the resistance of L1210/MTX may be attributable to the rapid recovery of dihydrofolate reductase, this explanation cannot be used to explain the insensitivity of P329. The P329 cell line actually had lower levels of enzyme activity than the drug-sensitive L1210 and recovered a slightly lower percentage of enzyme activity by 24 hours than did L1210. From the mouse studies, the conclusion was that, while the rate of MTX uptake was correlated with sensitivity to the drug, uptake correlated poorly with MTX inhibition of dihydrofolate reductase in these 3 cell lines.

Lack of Relationship Between Inhibition of Dihydrofolate Reductase and Deoxynucleoside Incorporation into DNA in the Mouse

The next approach with these 3 ascites tumor lines was to administer MTX and 2 hours later, before any detectable recovery of dihydrofolate reductase activity, to measure the *in vivo* incorporation into DNA of tracer doses of deoxyuridine or thymidine *(39)*. Under these conditions, with dihydrofolate reductase inhibited and the level of tetrahydrofolates possibly lowered by the 2-hour waiting period, MTX inhibited deoxyuridine incorporation by L1210 but not in the 2 drug-insensitive cell lines. With L1210, MTX increased the utilization of exogenous thymidine, but it did not significantly affect thymidine incorporation with the 2 other tumor lines.

One hour after the administration of doses of MTX which were 100 times greater than the optimal daily therapeutic dose and were in excess of the LD50 for the mouse, deoxynucleoside incorporation by the isolated ascites cells was not completely inhibited *(39)*. This was observed with cells obtained 1 hour after drug administration. From this study the conclusion was drawn that very little drug in excess of that bound to dihydrofolate reductase was retained in the washed, isolated cell. Advantage was taken of these observations to study in greater detail the action of MTX on deoxyuridine incorporation into DNA.

In Vitro Inhibition of Deoxyuridine Incorporation into DNA

MTX was administered to mice for inhibition of dihydrofolate reductase, and 2 hours later the cells were removed and incubated with deoxyuridine and with various concentrations of MTX. Under these conditions, deoxyuridine incorporation into DNA was increasingly inhibited by elevating the MTX concentration. A log-dose relationship could be demonstrated between the concentration of MTX and the inhibition of deoxyuridine incorporation into DNA. These studies indicated that drug, in excess of that required for the inhibition of dihydrofolate reductase, was necessary to inhibit deoxyuridine incorporation into DNA. They also suggested that the key reaction inhibited by MTX was some other site than dihydrofolate reductase.

A second site of MTX action has been suspected for a number of years. Folinic acid is a tetrahydrofolate derivative; as such, it is a product beyond the reaction catalyzed by dihydrofolate reductase. A competitive relationship should not, therefore, exist between the product of the inhibited reaction and the inhibitor. But, Hakala and colleagues observed a competitive relationship between folinic acid and MTX inhibition of the growth of Sarcoma 180 cells in culture *(18)* and an elevation in thymidylate synthetase activity in an MTX-resistant strain *(30, 42)*.

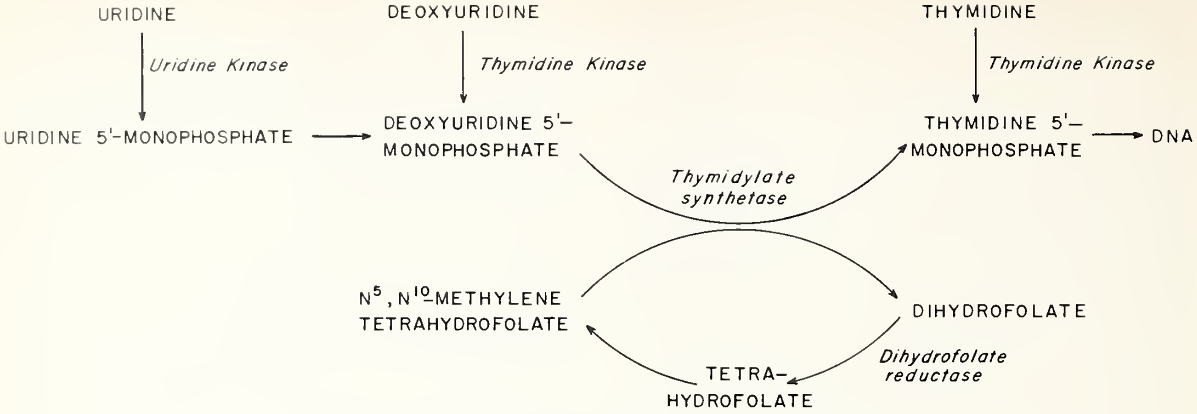
In addition, Wahba and Friedkin reported that MTX inhibited thymidylate synthetase, although at much higher drug concentrations than those required to inhibit dihydrofolate reductase *(43)*. Borsa and Whitmore, in a recent carefully detailed series of studies, emphasized the inhibition of thymidine 5′-monophosphate synthesis and thymidylate synthetase to explain the basis of L-cell sensitivity to MTX *(8–10)*.

Survey of Parameters Possibly Related to MTX Response

Our studies of human leukemias and the basis of MTX sensitivity were expanded to include the *in vitro* uptake of MTX by leukemic leukocytes and the examination (text-fig. 2) of *in vitro* deoxyuridine and thymidine incorporation as well as thymidylate synthetase, thymidine kinase, and uridine kinase *(38)*. The examination of deoxyuridine and thymidine incorporation into DNA permitted a study of thymidylate synthetase activity in the intact cell and provided a check on the cell-free enzyme assay. Thymidine kinase catalyzes the first step in the utilization of both substrates and provides cells with a reaction for scavenging these nucleosides *(11)*. Uridine kinase is a scavenger enzyme for salvaging uridine and conceivably might provide the cell with another avenue for bypassing and inhibition of dihydrofolate reductase.

We could not explain the variation in response to MTX on the basis of pretreatment levels of deoxynucleoside incorporation into DNA or the activity of thymidylate synthetase, thymidine kinase, uridine kinase, and dihydrofolate reductase. Leukocytes from patients with acute lymphocytic, acute granulocytic, or chronic myelogenous leukemia were similar with respect to these parameters *(35)*.

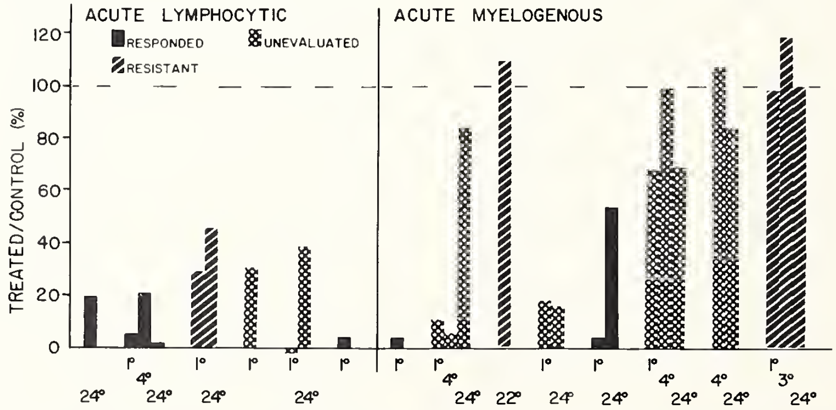
The role of MTX transport was less clearly defined for human leukemic leukocytes *(24)* than for the mouse transplantable ascites tumors *(25)* or for cell cultures *(17)*. MTX uptake by polymorphonuclear granulocytes exceeded uptake by leukocytes from patients with acute leukemia or chronic granulocytic leukemia. A greater than sevenfold range in the uptake of MTX was observed for leukemic leukocytes, with complete overlap in range for acute lymphocytic, acute granulocytic, and chronic granulocytic leukemias. There was a trend toward lower drug uptake by leukemic leukocytes from patients with MTX-resistant acute lymphocytic leukemia than for patients who responded to the drug. Presumably, there are factors which modify response to intracellular drug.



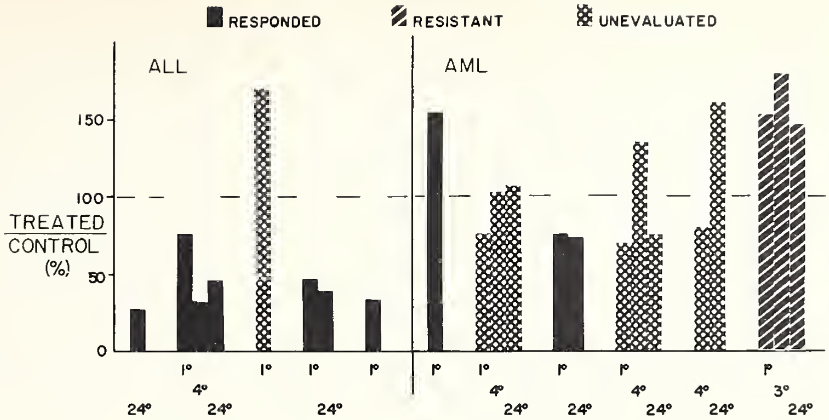
Text-figure 2.—Biosynthesis of thymidine 5′-monophosphate.

Deoxyuridine and Thymidine Incorporation

One of the first areas examined with an expanded approach was the relationship between MTX inhibition of dihydrofolate reductase and the effect of MTX on *in vitro* deoxyuridine and thymidine incorporation into DNA by human leukocytes. Leukocytes were obtained before and after MTX administration to patients with acute lymphocytic or acute granulocytic leukemias, and these 3 parameters were measured. There was no real difference in the inhibition of dihydrofolate reductase of leukocytes from these 2 groups of patients (text-fig. 1). However, the incorporation of deoxyuridine into DNA was much more sensitive to inhibition by MTX with leukocytes from children with acute lymphocytic leukemia than for adults with acute myelogenous leukemia (text-fig. 3). Deoxyuridine incorporation by lymphoblasts was more sensitive to MTX inhibition than the incorporation by myeloblasts. Deoxyuridine incorporation by 1 patient with acute lymphocytic leukemia, evaluated as clinically resistant to MTX, was still inhibited to the same degree as in myeloblasts from a patient considered sensitive to MTX. Again, for emphasis, more frequent and pronounced inhibition of deoxyuridine incorporation was noted for the 6 patients with acute lymphocytic leukemia than for the 8 patients with acute myelogenous leukemia. As with deoxyuridine, a more marked MTX inhibition of thymidine incorporation by leukocytes was generally observed for patients with acute lymphocytic leukemia than for patients with acute myelogenous leukemia (text-fig. 4). Thymidine incorporation was more frequently stimulated in acute myelogenous leukemia than in acute lymphocytic leukemia. The concentration of thymidine and of deoxyuridine in these studies saturated the rate-limiting step for incorporation into DNA.



Text-figure 3.—At various intervals after MTX administration, deoxyuridine incorporation into DNA by isolated leukocytes was assayed *in vitro* and compared with the pretreatment level of activity *(37, 38)*.



Text-figure 4.—At various intervals after MTX administration, thymidine incorporation into DNA by isolated leukocytes was assayed *in vitro* and compared with the pretreatment level of activity *(37, 38)*. ALL = acute lymphocytic leukemia; AML = acute myelogenous leukemia.

A depletion of the tetrahydrofolate pool of cofactors would presumably interfere with the synthesis of purine and pyrimidine precursors of DNA. The present observation of an inhibition of thymidine incorporation into DNA would be expected if purine precursors were depleted. The generally greater inhibition of thymidine incorporation with leukocytes from patients with acute lymphocytic leukemia than with acute granulocytic leukemia may indicate that depletion of the purine pool was less significant for DNA synthesis in acute granulocytic leukemia than for acute lymphocytic leukemia. Or, the two patterns may indicate that exogenous thymidine was diluted to a greater extent by endogenous thymidine in acute granulocytic leukemia than in acute lymphocytic leukemia. MTX inhibition of *de novo* thymidine synthesis would then increase the specific activity of exogenous thymidine incorporated into DNA as a result of blocking an intracellular dilution of labeled thymidine. The first interpretation would be at variance with the conclusions of Borsa and Whitmore *(8–10)*. The second interpretation would suggest that the leukocytes from patients with acute granulocytic leukemia may be less sensitive to MTX because of the greater availability of thymidine derivatives for these cells.

Drug-Induced Changes in Metabolic Pattern of Leukemic Leukocytes

The administration of drugs to patients with acute granulocytic leukemia caused pronounced changes in the enzyme pattern of leukocytes (table 1). Different patterns of changes in metabolic activity were observed after vincristine, MTX, and cytosine arabinoside (Ara-c). Variation occurred in response to administration of a drug to various patients, but changes in 2, or more, parameters frequently varied together. In one patient a set of parameters might be elevated, and in another patient after the same drug the set of parameters could be depressed. These variations and the clinical response of the patient have not yet been correlated.

Table 1.—Summary of correlations associated with drug administration to adults with acute granulocytic leukemia

Methotrexate *(36)*
1) Deoxyuridine incorporation and thymidylate synthetase

Cytosine arabinoside *(36)*
1) Thymidine and deoxyuridine incorporation
2) Thymidine kinase, uridine kinase, and thymidylate synthetase

Vincristine *(37)*
1) Thymidine and deoxyuridine incorporation, thymidylate synthetase, and dihydrofolate reductase
2) Thymidine kinase and uridine kinase

After MTX administration, a single coordinated change was observed *(36)*. The capacity of leukocytes from patients with acute granulocytic leukemia to incorporate deoxyuridine into DNA was related to an elevation in thymidylate synthetase activity and not to the recovery of dihydrofolate reductase activity. The elevation of thymidylate synthetase activity was associated with a greater recovery of the capacity to incorporate deoxyuridine into DNA. Different combinations of these 6 parameters were correlated after Ara-c and after vincristine.

After Ara-c, two sets of correlations were observed *(36)*. The inhibition of thymidine and deoxyuridine incorporation was related as might have been predicted. Thymidine kinase, uridine kinase, and thymidylate synthetase activity varied together. Dihydrofolate reductase activity varied independently of the two other groups.

After vincristine administration, deoxyuridine and thymidine incorporation, thymidylate synthetase activity, and dihydrofolate reductase activity simultaneously changed *(37)*. Changes in uridine kinase and thymidine kinase activity correlated but varied independently of the 4 other parameters. To reiterate, these drug-induced changes in metabolic patterns were both drug and patient specific.

The changes in enzyme activity which accompanied drug administration indicated a capacity for altering the metabolic pattern of the cell and were probably indirect results of drug action. An increase in enzyme activity may indicate the greater derepression of a gene within all cells or in a fraction of the population. Or, the increase may result from a stabilization of enzyme by either normal metabolites or drug products with a subsequent block of enzyme turnover. A nonspecific drug-induced lowering of enzyme activity may be the result of specific repression of enzyme synthesis or a general interference with transcription or translation. The observed multiplicity of correlations in these 6 parameters with administration of MTX, vincristine, and Ara-c requires the consideration of combinations of these control mechanisms plus the effect of changes in the cell population resulting from lethal drug action on sensitive leukemic cells.

CONTROL OF THYMIDINE KINASE ACTIVITY AND THYMIDINE INCORPORATION IN A HYBRID CELL LINE

The availability from Dr. Painter of a 5-bromodeoxyuridine (5-BUDR)-resistant subline (DFAF/BUDR) of a Chinese hamster cell, DFAF 28, provided an opportunity to study gene expression on some of these parameters *(41)*. A hybrid cell line, CSC, which originated from fusion of the 5-BUDR-resistant Chinese hamster subline, DFAF28/BUDR, and an Armenian hamster cell, 6B, permitted a study of the 2 parental genomes in the same cell. The hybrid cell contained the karyotype and chromosomal number expected for a cell with the total, or very nearly total, genetic complement of both parental lines.

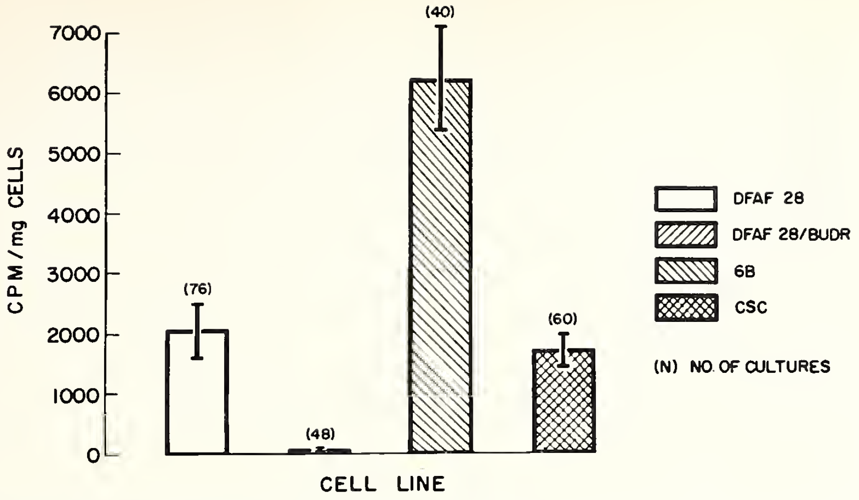
The incorporation of deoxynucleosides into DNA and thymidine kinase activity were studied in the ancestral Chinese hamster cell line, the 2 parental cell lines, and the hybrid cell line (text-figs. 5, 6). The 5-BUDR-resistant parental subline had low levels of thymidine kinase and was unable to incorporate thymidine or 5-BUDR into DNA. The original Chinese hamster cell line had approximately one-fifth the thymidine kinase activity and one-third the capacity to incorporate exogenous thymidine into DNA that was observed for the parental Armenian hamster cell line. The level of activity for these two parameters in the hybrid cell line was the same as that for the original Chinese hamster cell line, DFAF28, and had been carried by the 5-BUDR-resistant parental cell line, DFAF28/BUDR, although masked by the absence of thymidine kinase activity.

The hybrid cell expressed the presence of the Armenian hamster cell genome by thymidine and 5-BUDR incorporation into DNA, by overgrowth pattern, and by the presence of thymidine kinase activity. The level of thymidine kinase activity and the rate of incorporation of exogenous thymidine into DNA expressed the control mechanism present in the original ancestral Chinese hamster cell line, DFAF28. Presumably, the 6B genome for control of thymidine kinase activity and thymidine incorporation was present in the hybrid cell line, but the Chinese hamster cell genome for control of these parameters was dominant.

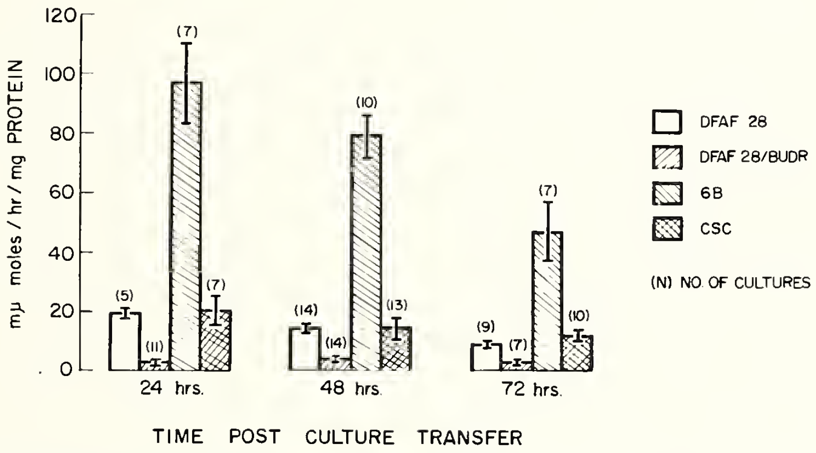
Hybrid cell lines provide a new experimental system for studying the control of enzyme activity; when the hybrid line is the product of 2 cell lines with variations in drug sensitivity, an additional tool may be provided for testing the relevance of a drug-induced alteration in metabolic pattern to variations in drug sensitivity.

USE OF A BIOCHEMICAL PARAMETER TO FOLLOW DRUG RESPONSE

The drug-induced changes in the metabolic patterns of leukemic leukocytes provide new ways for applying combination drug therapy. The correla-



TEXT-FIGURE 5.—Dwarf hamster cell lines and a hybrid cell line, CSC, were grown for 30 minutes in the presence of ^{3}H-thymidine-methyl, and the incorporation of radioactivity into DNA was measured *(41)*.



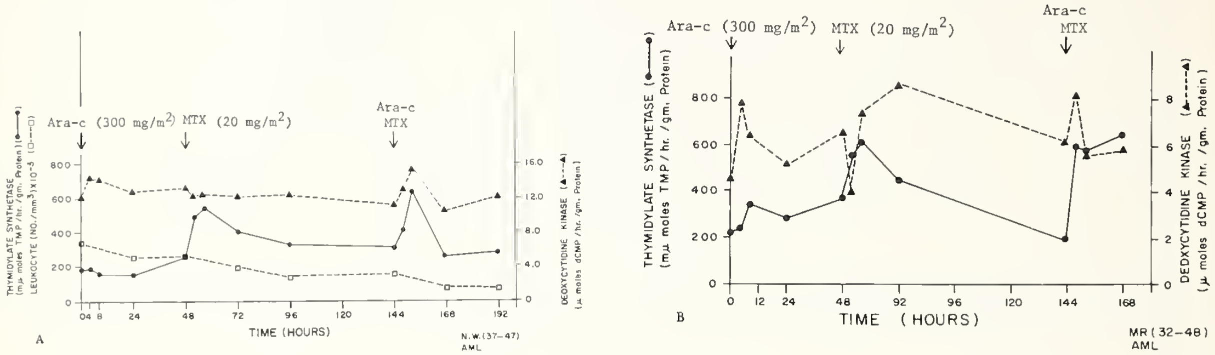
TEXT-FIGURE 6.—Thymidine kinase activity of dwarf hamster cell lines and a hybrid cell line, CSC, was assayed at 24, 48, and 72 hours after transfer of the cultures to fresh media *(41)*.

tion between the elevation of thymidylate synthetase activity and the capacity of leukocytes to incorporate deoxyuridine into DNA suggested that thymidylate synthetase activity contributed to recovery from MTX inhibition of DNA synthesis. If this assumption were correct, a block of thymidylate synthetase activity might increase the effectiveness of MTX.

Ara-c has some therapeutic effectiveness in the treatment of childhood acute granulocytic leukemia *(21)*. Ara-c decreased thymidylate synthetase activity in the leukocytes of some patients while it stimulated enzyme activity in other patients *(36)*.

For clinical reasons it was decided to study the therapeutic effectiveness of these 2 drugs as combination treatment for children with acute granulocytic leukemia. The changes in thymidylate synthetase activity would be followed for evaluation as a predictive index for selecting patients. Simultaneously, deoxycytidine kinase activity would be evaluated as a predictive index for sensitivity to Ara-c. The *in vitro* rate of Ara-c phosphorylation by intact cells predicted for the therapeutic response of mouse transplantable ascites cell tumors and human leukemias *(19, 26, 27)*. The response of these 2 enzymes to MTX and Ara-c, each drug administered separately and then in combination, was tested with leukocytes from 2 patients.

Ara-c alone did not affect leukocyte thymidylate synthetase activity in the first patient (text-fig. 7*A*), but caused a slight stimulation in the second patient (text-fig. 7*B*). MTX caused the expected elevation of enzyme activity in both patients. Thymidylate synthetase activity remained elevated for 3 days after MTX administration in 1 of the patients (text-fig. 7*A*). In combination therapy, Ara-c failed to block MTX stimulation of thymidylate synthetase. A partial response to these drugs by 1 patient was observed as a decrease in the white blood cell count (text-fig. 7*A*).



TEXT-FIGURE 7.—Activity of thymidylate synthetase *(37, 38)* and deoxycytidine kinase *(26)* in homogenates of leukocytes from 2 patients with acute myelogenous leukemia (AML) in relapse is expressed as μmoles thymidine-5′-monophosphate (TMP) or μmoles deoxycytidine-5′-monophosphate (dCMP), formed per hour per g protein. Ara-c and MTX were administered separately and in similar doses in combination. In 7*A*, the decrease in leukocyte count is also indicated. For the patient represented by 7*B*, the leukocyte count was 36,000/mm³ with 24% blasts at the initiation of the study and was 17,000/mm³ with 39% blasts at 24 hours after administration of the drug combination.

Deoxycytidine kinase activity was increased after Ara-c administration—either alone or combined with MTX. The other patient (text-fig. 7*B*) also had an increase in deoxycytidine kinase activity after MTX which was not observed in the first patient.

The elevation of the first enzyme in the activation of Ara-c may contribute to the greater efficacy of infusions over rapid intravenous administrations of Ara-c. An increase in deoxycytidine kinase activity by a sensitive cell in response to Ara-c may further increase the therapeutic index by increasing the rate of the initial reaction in drug activation. Of course, the final test of this approach to chemotherapy will be the correlation observed between the clinical response of patients to combination MTX and Ara-c therapy and a decrease in thymidylate synthetase activity resulting from Ara-c administration.

In CEM cell cultures, the level of thymidylate synthetase activity can be adjusted over a fivefold range by controlling the ratio of Ara-c concentration to MTX concentration. These cultures may provide an opportunity for developing an understanding of the biochemical basis for the changes observed in human leukemic leukocytes. With cell cultures, the Ara-c inhibition of thymidylate synthetase activity is more closely related to an inhibition of RNA synthesis than to that of DNA synthesis *(32)*.

DISCUSSION

There are some lessons to be learned from our experience with MTX. The observation of an enzyme inhibition by a drug does not necessarily mean that the mechanism of drug action is understood. Although the inhibition of dihydrofolate reductase is possibly one important facet in MTX action, it did not of necessity elicit a response to the drug. A comparison of MTX action on dihydrofolate reductase in drug sensitive and drug-resistant transplantable leukemias and human leukemic leukocytes indicated a lack of correlation with the variation in drug response. Elevated dihydrofolate reductase activity was not the basis of drug resistance in the patient and can only be considered important in the insensitivity of 1 of the 2 drug-resistant mouse cell lines.

Emphasis on the molecular basis of drug action has not, as yet, produced a predictive index for MTX sensitivity, and a trend has developed for reinvestigation of MTX action on intact cell preparations. In the study of intact cell preparations, the tendency to equate the rate of utilization of labeled thymidine with growth potential of the neoplastic disease must always be qualified. The efficiency of the incorporation of exogenous thymidine into DNA by a whole cell preparation depends on a minimum of 4 factors: 1) the number of cells in the preparation with the capacity to label DNA, 2) the precursor pool sizes and the rates of synthesis and degradation of the precursors in the pool, 3) the capacity of cells to convert thymidine to thymidine 5′-monophosphate, and 4) the rate of DNA synthesis. Frequently, there is the assumption that the rate-limiting reaction in the utilization of thymidine is DNA replication.

Utilization of tracer levels of exogenous thymidine to measure DNA synthesis may suggest an apparent stimulation of DNA synthesis as a result of the block of substrate dilution by *de novo* synthesis of thymidine 5′-monophosphate. All the present *in vitro* work on deoxynucleoside incorporation into DNA was with a concentration of deoxynucleosides that saturated the unknown, rate-limiting step. The decision to use this technique was based on the desire to minimize the dilution of labeled substrate by endogenous precursors. Although feedback inhibition has been demonstrated for pyrimidine biosynthesis, no attempt was made in the present studies to detect the possible development of feedback inhibition. But, high levels of precursors would be more likely to block *de novo* synthesis than would tracer levels of precursors.

On occasion, a stimulation of both thymidine and deoxyuridine incorporation, 24 hours after drug administration, has been observed *(36, 38)*. Presumably, the stimulation may have occurred more frequently but at a time that was not sampled. This elevation of deoxynucleoside incorporation may have resulted from an unrecognized block of pyrimidine synthesis, but, more likely, resulted from either a partial synchronization of the blood leukocytes in the S phase or a combination of DNA synthesis and repair with recovery from drug action. This period of increased incorporation of both deoxynucleosides may be an optimal time for administering drugs which interfere with DNA synthesis.

Although an inhibition of dihydrofolate reductase was observed after MTX, a complex, highly coordinated, and drug-specific response in the metabolic pattern of the human leukemic leukocytes was evident when the pattern for all the parameters was compared with the changes after vincristine and Ara-c. Chemotherapy may overcome these changes in metabolic patterns, or the alteration in patterns may have provided a compensatory way in which the effectiveness of chemotherapy was reduced. These changes in metabolic pattern do suggest new approaches and rationale for combination chemotherapy.

The relationship between the elevation of thymidylate synthetase activity and the recovery by leukemic leukocytes of the capacity to incorporate deoxyuridine into DNA indicated one possible approach for improving chemotherapy by combining Ara-c and MTX. In the initial studies, the 2 drugs were administered simultaneously for combination therapy, but the efficacy of this combination may depend on the selection of an optimal time for administration. The monitoring of changes in the activity of leukocyte deoxycytidine kinase and thymidylate synthetase after the administration of the individual drugs may provide information for the most effective temporal combination of the drugs. However, information is unavailable on whether Ara-c should be used to block the elevation of thymidylate synthetase or after the enzyme is elevated (on the basis that the cells are recovering from MTX inhibition and resuming DNA synthesis). If the latter event were more important, then monitoring deoxyuridine and thymidine incorporation would presumably contribute more useful information for scheduling Ara-c administration.

The observations on the hybrid cell line indicate some of the interactions that occur with 2 genomes in the same cell. Presumably, these types of interactions occur between dominant and recessive genes in malignant cells. The hybrid cell, with a karyotype similar to a summation of the 2 parental karyotypes, provides an opportunity to study these interactions. Of course, cell lines from an F_1 generation of homozygous parents should also be useful in pharmacologic studies, since cell lines derived from parental stock could be independently analyzed for drug response.

The suggestion of differences in the effect of MTX on deoxynucleoside incorporation into DNA by leukocytes from patients with acute granulocytic leukemia and acute lymphocytic leukemia may indicate the basis for the greater effectiveness of MTX in the treatment of acute lymphocytic leukemia. The explanation for these differences may arise from the variation in differentiation observed for the 2 leukemic cell types. Residual somatic controls in the leukemic cell may limit response to chemotherapy.

The inability to project a more clear-cut difference between sensitive and insensitive patients and between patients with different leukemias may have resulted from problems in measuring the response of patients to chemotherapy. Clinical criteria for patient response have been used to evaluate the sensitivity of the leukemia to MTX. These criteria may encompass too many variables not directly related to drug action or toxicity and may have resulted in the present difficulty of interpreting the data on deoxynucleoside incorporation.

New criteria clearly defining drug sensitivity are needed for pharmacologic studies *(12)*. The pharmacologic evaluation of a drug should not be based on criteria that fail to include some classification of the general physical condition of the patient. Conversely, a criterion based on decrease in cell number *per se* requires consideration of the general effects of the drug on normal cells. The establishment of adequate criteria will probably await the successful development of predicative indexes. Although many criteria will undoubtedly be tried, final evaluation will depend on the successful correlation of a few criteria of disease response with a pharmacologic or biologic parameter.

The clinical need for predictive indexes of tumor sensitivity is probably greater now than at any other time. The long-term survivals from acute lymphocytic leukemia resulting from combination-chemotherapy protocols provide strong evidence of the effectiveness of present drugs for 10% of the patients *(22, 29)*. The time lapse to complete evaluation of response of patient and termination of a study can exceed by 2–4 years the time required to secure and enter patients on protocol. Within this period, 2 or more new protocols will be initiated. An understanding of the metabolic basis for these long-term survivals should suggest drug combinations or dosage schedules which would lead to further improvement of chemotherapy for the remaining 85 or 90% of the patients. Desperately needed are criteria that will permit earlier evaluation of clinical protocols or suggest more effective ways of combining drugs.

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Predictive Tests in Culture of Drug-Resistant Mutants Selected *In Vivo*[1, 2, 3]

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MATERIALS AND METHODS

L5178Y cells grown in culture were maintained in the exponential phase of growth *(1)*. Cells were maintained in DBA/2 or AK/DBA/2 mice according to published methods *(1)*. For mutagenesis studies, a compact *(cpt)* clone was selected from the diploid murine leukemia cell line L5178Y, which in our soft-agar cloning method forms round, dense, tridimensional uniform colonies undistinguishable from L5178Y except for infectivity in DBA/2 mice. For growth in mice, 10^4 *cpt* cells were injected on successive weeks until takes were uniform when tested in 5 animals, each given injections of 10^3 cells. This subline was recloned and named *cpt a*.

To produce mutation in mice, 10^6 *cpt* cells were injected into DBA/2 mice. After 96 hours, the drug was injected subcutaneously, and the animals killed after an additional period of 96 hours. *Cpt* cells were recovered from the ascitic fluid, and the mutant frequency was determined in culture.

RESULTS AND DISCUSSION

The origin and properties of drug-resistant murine leukemic cell lines suggest that these cell lines originate by genetic mutation, whether *in vivo* or *in vitro*. Mutations to resistance to methotrexate (MTX) occur without the drug (table 1) *(1–5)*. MTX then provides a selective medium in which the reproduction of sensitive cells is prevented, but not that of resistant cells. Leukemic L5178Y lymphoblasts, whether selected *in vivo* or *in vitro*, indicate that a single mutational event can confer either a slight or a substantial alteration in sensitivity to MTX inhibition of cell reproduction in culture *(1–4)*. The data in table 2 indicate that "single-step" mutants, resistant to MTX, are diverse in origin and in the degree of resistance conferred. All mutants capable of growth at the highest level of drug are recovered equally well at the lowest level of MTX, and only a few mutants that grow at a low level of drug grow at the higher drug levels. At least 4 different degrees of resistance were found in this experiment. Biochemical mechanisms of resistance are increased levels of folic acid reductase activity *(2)*, or alteration of the transport mechanism for the drug *(3)*. Similar results are obtained when mutants resistant to cytosine arabinoside (Ara-c) or 5-iododeoxyuridine (IUDR) are recovered at increasing medium levels of the drug. A small increase in the level of drug results in a sharp decline in mutant frequency. Biochemical mechanisms of resistance to Ara-c are a partial or extensive deletion of deoxycytidine kinase *(4, 6)* or an increased pool of deoxycytidine phosphates *(5)*, possibly due to an increased level of nucleoside diphosphate reductase activity.

Resistance to IUDR is conferred by a partial *(6, 7)* or extensive loss *(7)* of thymidine kinase activity. Recurrent point mutations of a single gene would rarely produce the same structural alterations of

TABLE 1.—Spontaneous origin of mutants resistant to Ara-c *

Mutants per sample	Mutants per clone	
	Clones (expt. 1)	Clones (expt. 2)
9,7	8,5	2,10
11,9	5,7	2,0
12,11	5,20	0,0
13,13	27,12	6,1
11,12	3,0	1,0
10,5	3,18	3,22
16,13	18,13	122,4
11,6		
16,7		
Variance 7.5	96	970

* Twenty samples of 5×10^6 *cpt* cells per sample were evaluated for mutant number by incubation for 12 days with Ara-c (1.2×10^{-6} M) with the dilute agar colony method (col. 1, variance 7.5). The total cells from 14 (col. 2) and 15 (col. 3) subclones of *cpt* were incubated separately with the same level of Ara-c, and the variance was calculated.

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[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

[3] Supported by grants T-17D and T-17E from the American Cancer Society, Inc.

[4] The proficient assistance of Mrs. Barbara Morcaldi, Miss N. N. Tara, and Dr. Jana Raskova is gratefully acknowledged.

Table 2.—Change in mutant frequency with increasing levels of MTX*

M MTX × 10^{-8}	Mutant frequency†
3.6	240
5.2	40
7.2	8.0
11	2.2

*Cpt 4 cells in exponential growth were exposed to increasing levels of MTX, and the mutant colonies were counted after 12 days' incubation.

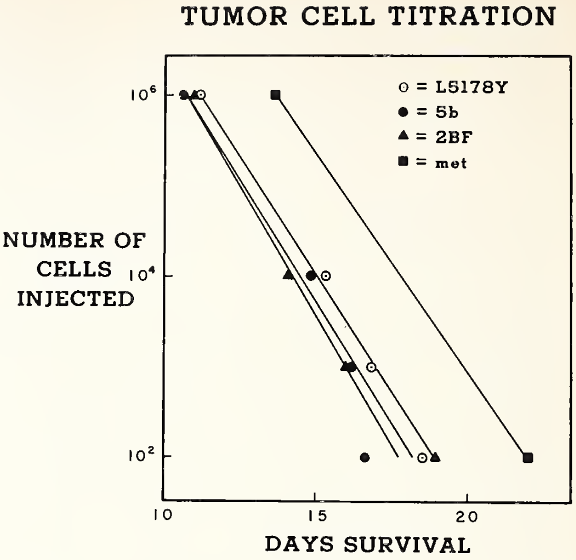
†Mutant frequency × 10^6.

DNA. Also, the determination of mutant frequencies permitted selection of a level of drug which would detect spontaneous mutation rates in the order of 1×10^{-6}. Thus, 1×10^{-6} cells from a subclone (20 generations) in 1 tube containing 5 ml of medium would provide about 1 mutant cell (table 3), a circumstance of maximum sensitivity, since spontaneously originating mutants are of lower average frequency in recently isolated subclones. Point mutations should be highly specific in biochemical consequences. L5178Y cells resistant to MTX *(3, 4)* or Ara-c are near-diploid and seem to have a chromosomal structure identical to that of the parent sensitive line *(5)*, suggesting that gross chromosomal alterations infrequently confer resistance in this cell line. Drug-resistant cell lines are usually unaltered in other aspects—cell volume, growth rate in culture, and sensitivity to other antimetabolites *(8)*. Infectivity in a genetically compatible host is altered slightly (text-fig. 1, table 3). A slight loss of infectivity in met cells occurs (MTX-resistant transport mutant clone, text-fig. 1), as well as a slight decrease in cloning efficiency in mice of the 5b clone [increased pool of deoxycytidine phosphate *(5)*] and the 2BF clone [extensive loss of thymidine kinase activity *(7)*]. Since infectivity in mice was retained by the mutant clones after 60 consecutive generations in culture, ample time is provided for sensitivity tests in culture of resistant clones originating in animals.

Table 3.—Percent cloning efficiency in mice and culture*

Cell line	Mice	Culture
L5178Y	52	70
5b	15	65
2BF	13	66

*Cells of each line were grown in culture by the soft-agar cloning technique. A minimum of 40 colonies was counted to provide the data given. In about 25 mice, 1 cell obtained by dilution was injected per animal, and cloning efficiency was calculated.



Text-figure 1.—Cells from each clone, grown in culture approximately 40 days, were injected into 15 first-generation AK/DBA/2 mice for each point, and the survival time in days was determined.

Point mutations, which govern sensitivity to antimetabolites, should also be demonstrable with the mutagenic agents, ^{137}Cs irradiation, ethylmethane sulfonate (EMS), and *N,N'*-nitrosomethyl guanidine (NTG) (table 4). In this work, a population of approximately 3×10^7 cells was generated from 10^2–10^3 cells to exclude pre-existing mutants from the inoculum. Equal parts of the population were exposed to the mutagen for 2 hours at a level sufficient to kill about 80% of the cells. To permit replication of mutant genes, cells were then incubated for 96 hours. Control cells, in parallel with mutagen-treated cells, were maintained in the exponential phase of growth. Mutants were detected by incubation of 1×10^6 cells per tube with the desired level of drug for 10–12 days in our soft-agar colony method. At 10–12 days, the colony size, approximately 10^4 cells, was readily detectable by 1.5-fold magnification in a Quebec Colony Counter. Control cells were manipulated in parallel with mutagen-treated cells. A minimum of 20 mutant clones was recovered to provide the data for any one determination.

For mutagenicity studies, it is essential to have available various genetic markers, since mutagenic agents exhibit selectivity for different genetic sites. The mutational markers available total 7, and we have obtained satisfactory biochemical data for 6 markers in the parent L5178Y cell line. Clones resistant to MTX, by virtue of an increase of the

TABLE 4.—Chemical mutagenicity of L5178Y cells: Mutant frequency × 10^6*

	IUDR (2.2×10^{-5} M)	Ara-c (1.2×10^{-6} M)	MTX (4.8×10^{-8} M)	Diffuse
Control	2.7	1.1	5.4	290
^{137}Cs	5.5	2.8	18.0	550
EMS	4.7	31.0	55.0	580
NTG	2.4	24.0	44.0	640

* Populations of *cpt* cells were treated with mutagen sufficient to produce 80% cell death (EMS = 2×10^{-3} M, NTG = 3×10^{-6} M) and grown for 4 days to permit reproduction of mutant genes. Mutants were detected by the soft-agar colony method.

level of folic acid reductase, are cross resistant to the antifolate pyrimethamine, and the MTX transport mutants are sensitive to pyrimethamine. Resistance to Ara-c, which occurs by a decrease in deoxycytidine kinase activity *(9)*, is unaltered in sensitivity to thymidine, and resistance to Ara-c, resulting from a mechanism that provides an increased cellular content of deoxycytidine 5′-phosphates, provides mutants which are cross resistant to thymidine *(5)*. Resistance to IUDR results from a partial or extensive loss of thymidine kinase activity *(7)*. Resistance to azauridine is attributable to a loss of uridine kinase activity *(7)*. The seventh mutational site is morphological, since it determines that the colonies formed will be larger, with irregular margins. These are called diffuse mutants.

Point mutational sites were found which govern sensitivity to MTX with ^{137}Cs irradiation, EMS, and NTG. Furthermore, some selectivity of mutagenic agents was found, since resistance to Ara-c was produced more readily by EMS and resistance to MTX by ^{137}Cs. Without definitive data (*e.g.*, transformation), it seems reasonable to assume that genetic mutation determines heritable sensitivity of leukemic cells to anticancer agents. Nongenetic refractory states determining responsiveness to drugs include stage of the cell cycle and rate of cell multiplication *(11, 12)*.

Various murine leukemic cell lines *(3, 5)* obtained directly from the host reproduce in culture with a high cloning efficiency (table 4, 50–90%)—a finding which establishes that the leukemia cells recovered in culture represent those present in ascitic fluids of the host. The same circumstance is found for L5178Y mutant derivatives resistant to MTX. Resistant leukemic clones, recovered from culture or from ascitic fluids during therapy, in most instances, quantitively retain their degree of resistance during extensive periods of reproduction in culture in the complete absence of antimetabolite (150 to greater than 1,000 consecutive cell generations), demonstrating that growth tests in culture of the degree of resistance of leukemic cell populations obtained from the host can be accomplished in a dependable manner *(3, 5)*. The leukemic cells from culture are only slightly unable to produce leukemia when reinjected into genetically compatible hosts *(3, 4, 10)*.

Although apparently drug sensitivity of leukemic cells *in vivo* can be quantitated *in vitro,* agents which require activation by the host before antileukemia effectiveness might inhibit cells in culture by a mechanism unrelated to that found *in vivo,* and drug-resistant clones originating in the host may not be capable of reproducing in culture. Single anticancer agents can affect various biochemical targets in the cell, and the mechanism responsible for cell death has rarely, if ever, been determined. Of 18 MTX-resistant clones recovered during therapy in mice, only 11 involved increased levels of folic acid reductase, suggesting that drug-sensitive target sites other than folic acid reductase can determine responsiveness *in vivo.* An understanding of host-related factors which determine drug sensitivity (variable drug levels, different nutritional circumstances) should be possible in systems that provide a high cloning efficiency in culture of cancer cells obtained from the host.

From these studies of mutagenicity of L5178Y cells in culture and demonstration of the infectivity of the leukemic cells in genetically susceptible hosts, it is evident that a host-mediated assay of mutagenic agents on near-diploid mammalian cells in the host can be done. Thus, using single EMS injection of either 8, 6, 4, or 1 mg, the MTX mutant frequency in cells recovered from mice has been increased to 22×10^{-6}, 9.6×10^{-6}, 6.4×10^{-6}, and 1.8×10^{-6}, respectively, compared to the saline-injected control value of 1.1×10^{-6}. Thus, a new and practical method has been developed for the evaluation of mutagenic agents for somatic mammalian cells in a host-mediated system.

SUMMARY

Murine antimetabolite-resistant leukemic lines, originating from L5178Y *in vivo* or *in vitro,* are presumably genetic in origin. Nongenetic drug-refractory states include state of the cell cycle and rate of cell multiplication. Circumstances which permit the near-quantitative recovery from the host of leukemic cells capable of continuous reproduction *in vitro* can provide a simple sensitivity assay in culture predictive of tumor responsiveness *in vivo*. Exceptions would include drugs activated by the host (*e.g.,* cortisol) which would inhibit cells in culture by a mechanism independent of that found *in vivo*. Anticancer drugs exhibit various biochemical effects on the cell, and the mechanism by which cells are killed has not been established. Consequently, the variable levels of drugs, circumstances of nutrition, and sites of tumors which influence responsiveness *in vivo* could be investigated with culture methods when leukemia cell-cloning efficiency in culture is high (40–90%).

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DISCUSSION

Chairman: I am not too surprised with the results of this Conference, which might tend to discourage us in the use of chemotherapy rather than encourage us. I certainly have been impressed over the years with the fact that drugs we have used in solid tumors are pretty good. The trouble is that the response rate is so small and the recurrence rate so rapid; hence, the precise killing value of the drug is lost in our gross estimates of how good drugs are in clinical patients.

Dr. Bertino: I would like to ask Dr. Fischer whether he can now give us some information on whether or not chemotherapy combinations have the potential of eliminating persistent classes of drug-resistant cells.

Dr. Fischer: A system like this has a powerful advantage because we can manipulate drug levels, sequence of drug treatment, time of exposure in sequential use of the drugs, and pretty quickly sort out some of the parameters, at least as far as the whole cell is concerned. Methotrexate (MTX) sensitizes cells beautifully to cytosine arabinoside (CA), not the reverse; the rate of cell death is more rapid by a factor of 2 logs, after only 1 hour of pretreatment with MTX. Incidentally, this does not fit into any neat cell-cycle kinetic model that I can think of.

Dr. Mihich: I wonder whether Dr. Fischer has information on the cloning efficiency *in vivo* in host animals which are pre-irradiated. I am referring particularly to that line which seems to be shifted to the right. Might that be the result of having corrected the line which is more antigenic? There are 2 examples I know of in the literature of reducing the sublines which are more antigenic than the parent line. One is a report in a talk by Dr. Hutchinson, which she gave here last summer, that a resistant subline is clearly more antigenic than the parent line. Is this preselection or is this mutation?

Dr. Fischer: We would have to reisolate a similar mutant again. It should not be too difficult to select from the animal during treatment an MTX-resistant clone by virtue of transport, to reinject it immediately, and to see if it titrated in a different manner.

Dr. Wilmanns: I have a few questions for Dr. Roberts. First, I wonder that you never find increased levels of dihydrofolic reductase in leukemic cells under the action of MTX; we have found this increase in most of the phases. Do you not think that the thymidylic synthetase is therefore important in resistance because this is a limiting enzyme in the thymidine methyl group synthesis? Second, do you know anything about the nature of the increased activity of thymidylic synthetase under the action of MTX? Is it possible that synchronization of the cells causes this increased activity? Or is it possible that the concentration of tetrahydrofolic acid by itself influences the activity of the thymidylic synthetase? Also, is it possible to reverse the effect of MTX on thymidylic synthetase by addition of the citrovorum factor? Finally, why do you not combine the MTX treatment with 5-fluorouracil (5-FU) treatment because this is a specific inhibitor of thymidylic synthetase?

Dr. Roberts: I do not see an elevation of dihydrofolic reductase after MTX therapy. We have looked at groups of patients at different stages in their therapy and after they had courses of therapy, which would put them several months away from MTX. Even then we did not find the elevation in acute leukemia. The working hypothesis I am following right now is that this elevation of thymidylate synthetase activity may be important in the recovery from MTX. I would have liked to use 5-FU but I was afraid I could not get clinicians to agree to its use. I selected CA as a drug, for clinicians were interested in it. We had shown already that, at least in some patients, it severely inhibited thymidylate synthetase. Citrovorum factor did not, in cultures of a human cell line derived from a patient with acute lymphocytic leukemia, cause elevation of thymidylic synthetase activity, nor did thymidylate synthetase reverse the elevation caused by MTX.

Dr. Hall: In the host-tumor relationship, you have 2 or 3 genetic systems going, all of which can contribute to states of sensitivity and resistance and any one of which could be exploited in the selection of drugs. The two major times that resistance has to be considered are initially, and when it develops (acquired resistance) under the pressure of drug administration. Certain drugs metabolize by pathways genetically determined by the host; *e.g.*, the deletion of pyrophosphorylases give rise to metabolic diseases such as the Lesch-Nyhan syndrome. These not only characterize the marrow where a large amount of DNA is constantly made but host cells in general; *e.g.*, you can see this defect in explants of skin. The initial incapacity of a patient to respond to 5-FU may be due to deletion of pyrimidine phosphoribosyl transferase which is not specifically characteristic of the tumor but may be a genetic characteristic of the host. I do not know whether anyone has ever looked into this but it should be investigated because it occurs in other diseases. In a patient with breast cancer, it is difficult to analyze a piece of tissue from a lump in the middle of the thorax to decide whether you are going to treat it with 5-FU. It would be simpler if initial resistance were genetically determined for the whole host, since you could analyze a piece of gingiva or buccal mucosa. There is a need to formulate a prediction system that might relate to the genetic state of initial drug resistance of the host.

Dr. Fischer described the constant mutations that occur in the highly unstable genetic system of the tumor parasite living within the host. This is important both as to whether or not the tumor is going to be initially sensitive and also very important in acquired resistance, since at least 50% of the 85% who respond will relapse in time. There can be several mechanisms for this resistance. Our group has found that, in a specific patient with acute lymphocytic leukemia, no single genetic determinant can be repeatedly shown to be the single cause of resistance. A patient can have at one time a highly sensitive acute lymphocytic leukemia population and later on have a highly drug-resistant population genetically. However, he always has genetically constant populations in the bone marrow cells and gut mucosa that never really change with respect to drug resistance. Does the normal cell population of the host not mutate into a drug-resistant type because *(a)* there is some inherent genetic stability of the host tissue; if this is so, can it be exploited, or defined? Or *(b)* because it has to do with something else, such as a very low rate of cloning efficiency? For example, in normal marrow, imagine an emergent mutant clone of normal granuloblasts resistant to MTX but having cloning efficiency *in vivo* that is immediately lowered by contact inhibition. If this is true, could you apply everything you could learn about poor cloning efficiency in normal marrow and try to establish the same set-up in the malignant situation?

Dr. Bertino: It would not necessarily have to be what we think of as chromosomal change but a somatic change in leukemic cells, *i.e.*, a selection, in some way, for a cell type which was repressed in a different manner. The other point is you do have a tremendous cloning efficiency exerted in the patient by the toxicity and the need for regrowth of the marrow element. So, I think this would suggest an opportunity for tremendous pressure.

Dr. Fischer: I have heard of attempts to demonstrate mutations of normal cells in mice, presumably to resistance. It is a rather expensive experiment and involves the use of irradiated animals in the case of the marrow, but, I think it would be a very important study. It is true that the turnover of the malignant marrow would be more rapid in the leukemias and therefore we would have drug-resistant cells emerging.

Dr. Hall: I would like a definition of what we are considering mutation here. I wonder if it is not like one end of a bell-shaped curve of enzyme distribution that activates drugs within these different cells and more clones form with lesser or greater concentrations. The second point is there may emerge resistant clones in normal tissue which may be what enables us to give repeated courses of chemotherapy without killing off the bowel mucosa or bone marrow completely. I think there is the same kind of emergence of resistance in normal cells as there is in leukemic cells.

Dr. Bertino: I was expecting Dr. Skipper to comment that normal bone marrow and gastrointestinal mucosa can probably only tolerate 10^2 or 10^3 logs of kill, so that the chances of getting a resistant mutant are very slim just on a mathematical basis. Whereas when we are considering tumors that may have 10^9 or 10^{12} cells, the chances of encountering a resistant clone are very great.

Dr. Ellison: The other problem in deciding whether there is a resistant mutant in the normal cells of the human host is that, as the disease progresses and reaches the point of resistance in leukemic cells, whether it is for metabolic reasons or just geographic reasons, there are more tumor cells around and apparently fewer normal cells left to proliferate. So we see, in leukemic patients at any rate, more anemia and more thrombocytopenia. We had a number of patients with chronic granulocytic leukemia who were receiving 6-mercaptopurine (6-MP) intermittently. Over and over again, we could reduce the abnormal cells or reduce the count and spleen size with a predictable amount of 6-MP or chloropurine without observing much of a reduction in platelets or hemoglobin. As we proceeded, we saw increasing sensitivity of the platelets and of the red cells, and it did not seem to be a matter of progression of disease. We stopped using these drugs to go back to busulfan or spleen radiation and did not see this to the same extent. So this raises the question of whether there was increasing sensitivity in the normal cells.

Dr. Fischer: One thing that I forgot to say was that the cells we isolated in culture were in two instances with MTX and in four others with antimetabolites resistant in the animal to therapy with the drugs. On the question of the diversity of the population, in the increasing mutants that you can see toward the bottom end, the mutants are diverse because there are many different levels of drugs. Each one is a clone, and not many of them would be alike. If you had a mutation rate of 1×10^{-6} and the population went from a million to a billion, you would have many different kinds of mutants present—many different kinds because genetic mutation does not that frequently repeat itself as a spontaneous effect.

PANEL V

STUDIES IN LEUKEMIA

Relevance of *In Vitro* Tests for Predicting Responsiveness to Antitumor Agents[1, 2]

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ONE of our major goals was to improve chemotherapy of malignant disease, particularly leukemia. We sought specific determinants of responsiveness to different antineoplastic agents, with a view toward the design of chemotherapeutic programs individually relevant to each patient. Without such procedures, drugs must be chosen on the basis of statistical considerations—the results of large-scale clinical trials with each drug.

Predictive procedures are important for rapidly assessing probable drug responsiveness. In acute myelogenous leukemia, unless remission is achieved rapidly, the patient may die before a second empirical therapy can be tried. Therefore, it is important to avoid the use of an ineffective drug or drug combination, since the success of any chemotherapeutic program depends partly on the rapidity with which the most appropriate drugs can be selected.

In the past, many predictive tests for selecting antineoplastic agents were developed. None is in common use today. Most of these procedures depended on the ability of the investigator to achieve growth or limited survival of human tumor cells in tissue culture or animal hosts. This is not yet feasible on a routine basis without extensive supporting facilities. We have therefore sought determinants of drug responsiveness that do not depend on maintenance of *in vivo* growth but utilize freshly isolated, malignant cells.

Determinants of Drug Responsiveness

The ultimate measure of drug responsiveness is drug-promoted survival of tumor-bearing hosts. Such a determination can readily be made in animals; in man, responsiveness is measured by remissions, regressions, suppression of production of leukemic cells, and related parameters. Such measurements involve drug administration, followed by careful observation of the subsequent course of the neoplastic disease.

Relevance of Studies on Animal Neoplasms

The animal neoplasms, particularly transplantable murine leukemias, provide the investigator with a ready source of neoplastic cells of known drug responsiveness. Cell lines showing a spectrum of responsiveness to each major drug are usually available. Using such lines, we can investigate the nature of determinants of drug responsiveness. We consider that a test procedure unable to distinguish between drug-resistant and drug-sensitive animal tumor cells will probably be unable to make such a distinction when human malignant cells are involved.

Types of Drug Resistance: Permeability Barriers

The most striking example of the role of permeability barriers as determinants of drug responsiveness is found with methotrexate (MTX). The capacity of freshly isolated cells for MTX uptake was found related to drug responsiveness in both animal *(1)* and human *(2)* leukemia cells. Levels of the drug-sensitive enzyme, dihydrofolate reductase, were increased in examples of drug-induced resistance *(3)* but not in inherent drug resistance *(4)*. Among the normal cell types, granulocytes and platelets had a high capacity for MTX uptake; normal lymphocytes and erythrocytes were relatively resistant to MTX. Although the measurement of uptake can predict for drug responsiveness in both human and murine leukemias, it is noteworthy that drug accumulation by the most responsive human cell type was slower than drug accumulation by the most resistant murine leukemia (text-fig. 1). Uptake as a determinant of responsiveness must, therefore, be considered on a species-specific scale of units.

Other examples of permeability barriers to drug absorption have been implicated in the development of resistance to methylglyoxal-bis-guanyhydra-

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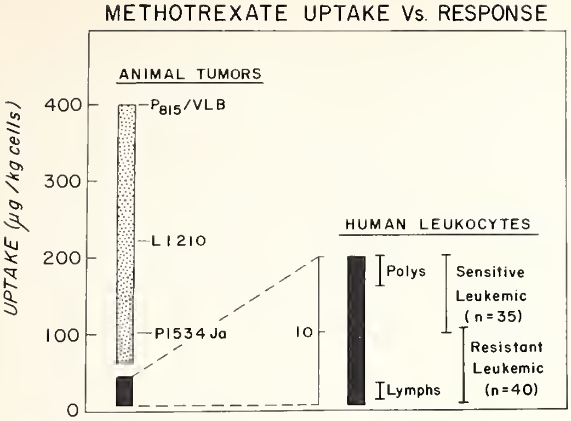
[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

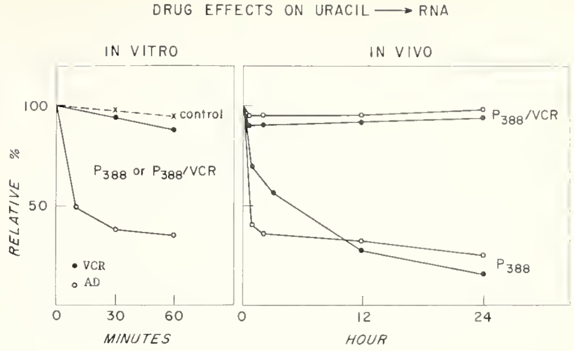
[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.



Text-figure 1.—MTX uptake by animal and human leukemias. Cells were incubated in medium containing 100 µg/liter. Data are given in terms of drug uptake over a 15-minute interval by transplantable animal leukemias and by human leukemic cells. Drug uptake by normal human polys (granulocytes) and lymphs (lymphocytes) is also shown.

zone *(5)*, to actinomycin D (AD) *(6)*, to certain terephthalanilides *(7)*, and to daunomycin *(8)*. For the latter 3 agents, such barriers could only be demonstrated *in vivo* or in cells growing in culture. Our attempts were unsuccessful to predict for responsiveness to AD or daunomycin, solely on the basis of *in vitro* measurements of uptake *(6, 8)*. The data indicated that these agents readily penetrated drug-sensitive and drug-resistant cells, but that barriers to drug-DNA interaction were maintained in the latter cells during growth. When cells were placed in a medium that did not permit growth, these barriers were rapidly lost, and all cell types became equally sensitive to drug-induced inhibition of, for example, RNA synthesis. Text-figure 2 shows the results of a study on 2 cell lines varying markedly in responsiveness to AD: P388 (sensitive) and P388/VCR (resistant). AD inhibited incorporation of uracil into RNA by P388 *in vivo*, but it inhibited such incorporation by both cell types *in vitro*. Interestingly, vincristine (VCR) also inhibited incorporation of RNA formation by P388 *in vivo*, but it did not do so *in vitro* in either cell type.

The alkylating agents, busulfan and nitrogen mustard, readily penetrated both drug-resistant and drug-sensitive murine leukemia cells *(9)*. These 2 agents readily inhibited *in vitro* nucleic acid synthesis by both drug-resistant and drug-sensitive cell types.



Text-figure 2.—Inhibition of incorporation of uracil into RNA. *Left:* Tumor cells were incubated in medium containing VCR (5 µg/ml) or AD (0.5 µg/ml) for specified intervals, samples were withdrawn, and the capacity of the cells for incorporation of uracil into RNA was measured. Results were identical when P388/VCR was substituted for P388. *Right:* Therapeutic doses of AD (50 µg/kg) or VCR (250 µg/kg) were injected intraperitoneally into animals bearing advanced P388 or P388/38280. At specified intervals, cells were removed and resuspended in medium, and their capacity to incorporate uracil into RNA was measured.

Phosphorylation and Responsiveness

When we measured transport of the fluorinated pyrimidines *(10)*, 6-mercaptopurine (6-MP) *(10, 11)* and cystosine arabinoside (Ara-c) *(12)*, we found that these agents readily penetrated all cell types, regardless of the relative drug responsiveness of the cell. But the capacity of the different cell types for phosphorylation of these agents varied markedly. In transplantable murine leukemias, responsiveness to 5-fluorouracil (5-FU) *(13)*, Ara-c *(14)*, and 6-MP *(11)* was closely correlated with the capacity of freshly isolated, intact cells to convert these agents to nondiffusible forms (mainly drug nucleotides). An association between drug conversion to nondiffusible metabolites and drug responsiveness was also found in human leukemias for 6-MP *(11)* and Ara-c *(12)*. Most human leukemias are unresponsive to 5-FU and show little capacity to phosphorylate the drug *(15)*.

We also compared the rate of drug conversion to nucleotides with drug responsiveness in human and murine leukemias (text-fig. 3). The range of phosphorylation of most of these compounds was similar in the 2 species. The data on 5-fluorouridine (FUR) and 5-fluoro-2′-deoxyuridine (5-FUDR) were provided for comparison. The capacity for phosphorylation of these agents *in vitro* did not provide a basis for prediction of drug responsiveness.

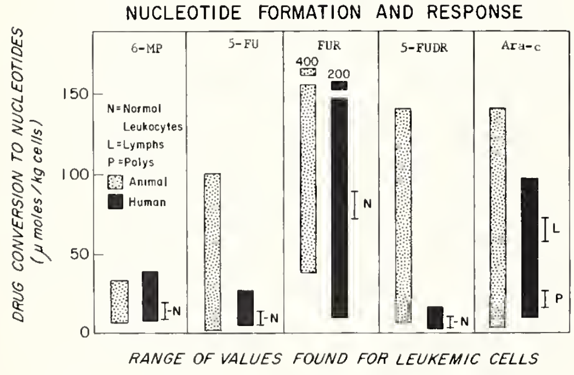
Procedures Involving Drug Administration

For certain agents, *e.g.*, nitrogen mustard and AD, *in vitro* procedures have not been useful in making distinctions between drug-resistant and drug-sensitive cell types. We could, however, make such distinctions by a procedure involving drug administration to leukemic mice or to patients with leukemia. Leukemic cells were isolated just before, and at intervals after, administration of a therapeutic drug dose, and the capacity of the cells to incorporate labeled uridine and thymidine into RNA was measured. If drug administration caused a profound loss of such capacity, the leukemia was usually found drug responsive. An example of the data obtained with the transplantable murine leukemias is shown in text-figure 2. Our data on leukemic patients will be summarized elsewhere.

Enzymatic Determinants of Drug Responsiveness

Resistance to Ara-c has been associated with a fall in levels of deoxycytidine kinase *(16–18)*; both uridine kinase and uridine phosphorylase have been implicated as determinants of responsiveness to the fluorinated pyrimidines *(19, 20)*, as has thymidine kinase *(21, 22)*.

Levels of inosinic pyrophosphorylase were found related to responsiveness to 6-MP in some *(23)*, but not in all *(24)*, human leukemias tested. The prospect of measuring levels of particular enzymes as a means of predicting drug responsiveness is attractive, since such procedures could presumably be applied to leukemias and to solid tumors, and the need for freshly isolated cells would be eliminated. Our studies along these lines were directed toward the fluorinated pyrimidines and the group of drugs for which resistance appears to be based on *in vivo* permeability barriers (AD and the vinca alkaloids).



Text-figure 3.—Capacity for nucleotide formation by animal and human leukemias. Cells were incubated in medium containing 0.1 mM drug. Data are given in terms of drug phosphorylated during a 15-minute incubation. N = normal human leukocytes; L = normal small lymphocytes; and P = normal polys (granulocytes).

Initially, we found that responsiveness to 5-FU could be correlated with capacity of freshly isolated cells to carry out phosphorylation of the drug *(13)*. But certain cell types showed only limited capacity for such phosphorylation and were markedly responsive to 5-FU *(25)*. In addition, supplementing 5-FU therapy with glucose or inosine promoted drug phosphorylation *in vivo* but only led to increased drug toxicity *(26)*. Thus the measure of total drug phosphorylation seemed to be only an index of drug responsiveness, not a specific determinant thereof. Reyes and Hall *(27)* showed that levels of a pyrimidine-5′-phosphorylribosyl transferase (which directly forms 5-fluorouridylic acid from 5-FU) were correlated with 5-FU responsiveness in murine leukemias. The presence of such an enzyme accounts for 5-FU responsiveness in the P388/38280 *(25)* and in the LPC-1 murine plasma cell tumor *(28)*, both of which have negligible levels of uridine phosphorylase and, therefore, have impaired capacity for phosphorylation of 5-FU. In addition, we found that levels of thymidine kinase were highest in murine leukemias inherently resistant to 5-FUDR *(22)*. The latter enzyme provides a pathway for biosynthesis of thymidylic acid which circumvents the 5-FUDR-induced blockade of thymidylate synthetase *(29)*. Perhaps a study of levels of thymidine kinase and the phosphoribosyl transferase might predict for responsiveness to 5-FU and 5-FUDR in extracts of neoplastic cells.

In other studies, we found that alterations in levels of enzymes responsible for metabolism of membrane and secreted glycoproteins were associated with development of resistance to AD and the vinca alkaloids *(30, 31)*. The data of table 1 show the results of comparisons of several such cell lines. Development of resistance to these drugs was accompanied by major alterations in representative enzymes involved in formation and degradation of bonds between sugar moieties on the carbohydrate portion of the glycoprotein molecule. Development of resistance to other drugs, *e.g.*, 6-MP, Ara-c, and 5-FU, caused no such alterations.

TABLE 1.—Glycosidase activity in murine leukemia cells* and activity of glycoprotein transferases in murine leukemias†

Enzyme	L5178Y	L5178Y/D
Glycosidase acitivity		
Fucosidases	0	0
β-Xylosidase	2	1
α-Mannosidase	9	4
α-Glucosidase	10	3
β-Glucosidase	14	8
α-Galactosidase	28	18
β-Galactosidase	74	31
N-β-Acetylgalactosaminidase	26	10
N-β-Acetylglucosaminidase	260	100
Acid phosphatase	206	209
Activity of glycoprotein transferases		
Collagen : glucosyl	320	780
Collagen : galactosyl	2130	3619
Glycoprotein : galactosyl	770	1470
Fetuin : fucosyl	680	1111
Porcine submaxillary glycoproteins : fucosyl	440	972
Fetuin : *N*-acetylglucosaminyl	239	260

* Units: mμmoles/mg protein/hour.

† Units: counts/minute/mg protein.

Current Methodology

We routinely obtained leukemic cell samples (100–500 mg) from patients who were candidates for chemotherapy. Tests on drug uptake and phosphorylation were done on a total of 40 mg of cells. Tests for the capacity of these cells for conversion of labeled uridine deoxyuridine and thymidine to nucleic acids were done on another 60 mg of cells. Cell samples, obtained after initiation of a therapy program, were used for subsequent measurements of DNA and RNA formation. Homogenates of leukemic cells and of solid tumors were used for measurement of deoxycytidine kinase, phosphoribosyl transferase (substrate = 5-FU), and membrane glycosidase and glycoprotein transferase activity. These assays required a total of 100 mg of cells.

Conclusions

Our current procedures for prediction of responsiveness to MTX, 6-MP, and Ara-c were useful in selection of drug-responsive patients *(2, 11, 12)*. The procedure, involving *in vitro* measurements of nucleic acid synthesis after drug therapy, enabled us to evaluate the probable results of other drug therapy. Enzymological studies on determinants of drug responsiveness provided a means of distinguishing among animal neoplasms varying widely in drug responsiveness; the relevant human studies have not been completed.

Apparently, only a limited number of predictive tests may reach widespread use. But an important side effect of our studies is an increase in our understanding of the basis for the action of the antineoplastic agents and of the basis for the development of drug resistance.

SUMMARY

Selection of agents for antitumor chemotherapy is usually made on the basis of empirical considerations. In our studies on modes of drug resistance, we developed *in vitro* tests which aided in the evaluation of probable responsiveness of human antineoplastic disease to different drugs. Statistical studies indicated that such tests are valid for 4 clinically useful agents: MTX, 5-FU, 6-MP, and Ara-c. Similar procedures should be equally useful for examination of other agents for which cellular uptake or conversion to ionized products is the major determinant of responsiveness.

The current procedures required 25–50 mg samples of intact, freshly isolated leukemic cells or cells from malignant effusions. Drugs for study had to be available in radioactive form. The capacity of cells for drug uptake or for conversion of drugs to nondiffusible forms was measured. Data on cell samples from patients with known degrees of drug responsiveness were available for evaluation of results on new patients.

For another group of drugs, drug-resistant and drug-sensitive cell types could not be distinguished in an *in vitro* examination of drug uptake rates, binding to cell components, biotransformations, and drug-induced inhibition of protein or nucleic acid synthesis. Both murine and human leukemia cells were used. This group of drugs included the alkylating agents, the vinca alkaloids, AD, daunomycin, and the phthalanilides. Resistance to certain of these agents involves barriers to drug-DNA interactions, which are only demonstrable *in vivo* or *in vitro*. Recently we found that such resistance is also asssociated with alterations in levels of enzymes responsible for glycoprotein metabolism. Altered membrane glycoproteins could account for

changes in permeability of outer and inner membranes of tumor cells.

Certain classes of glycoproteins contain L-asparagine, bound to a carbohydrate moiety. Treatment of murine leukemia cells with L-asparaginase inhibits production and enhances degradation of such glycoproteins; the extent of these effects can be correlated with L-asparaginase sensitivity in the various tumor cell lines tested *(32)*.

Certain tests described above can be done on cell homogenates and would, therefore, be appropriate for studies on solid tumors. Enzymatic determinants of responsiveness to other agents have also been elucidated. Levels of deoxycytidine kinase, pyrimidine-5′-pyrophosphorylase, and thymidine kinase were correlated with degrees of responsiveness to Ara-c, 5-FU, and 5-FUDR, respectively, in murine leukemias. These determinations are suggested as possible means of assessing drug responsiveness of homogenates of solid tumors.

For certain agents, *e.g.*, cytoxan, we have no suitable *in vitro* predictive test. Such agents, as well as other drugs discussed above, could be evaluated by administering a single therapeutic dose to patients with leukemia and obtaining blood samples just before and at 24- and 48-hour intervals after drug administration. Capacity of isolated cells for incorporation of deoxyuridine and uridine into nucleic acids could be measured *in vitro*. A significant decrease in the extent of this incorporation was generally associated with drug responsiveness. This test is not particularly suitable for studies on solid tumors or on patients with low numbers of peripheral leukemic cells. In the latter case, marrow samples can be successfully used.

A combination of *in vitro* tests and *in vitro* examination of nucleic acid synthesis following a drug pulse is currently in use in our laboratories. This procedure permits rapid evaluation of probable responsiveness of patients with leukemias to several potentially useful agents. Such tests have also been routinely applied to cells from malignant effusions. The relevance of enzymatic determinations of drug responsiveness of solid tumors is currently under evaluation.

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Selecting Drug Regimens in Human Leukemia[1, 2, 3]

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WE ALL hope that the empirical search for new anticancer drugs, so productive in the past, will turn up even more effective agents against specific neoplastic diseases or find a fundamental defect common to all neoplasms. However, this search should not blind us to the fact that many effective drugs are now available to our patients. One of the most important protocol problems that an oncologist faces is selecting, out of a number of possible drugs, the few that may help his patient. At the present time of the enthusiastic use of multiple (up to 7) drug combinations, another need is to omit those drugs not of benefit to a particular patient, thus saving him from unnecessary toxic effects. An approach to these problems is easiest in the acute leukemias for many reasons: 1) There are more drugs effective against acute leukemias than against solid tumors; 2) within these leukemias, one major type, lymphatic, is very drug sensitive, while the acute myelogenous type is resistant; and 3) sampling neoplastic cells is simpler for acute leukemias than for solid tumors; especially when recurrent neoplasms develop, the solid tumors are apt to be deep-seated and inaccessible for sampling.

METHODS

To be effective, antileukemia drugs must be taken up by, and retained within, leukemic blasts and, when there, they should inflict lethal damage on the target cells. For a predictive test to be useful, it should measure such effects within the first 7 days of therapy. To measure these effects, we have designed a number of tests which follow the unique characteristics of each potential drug.

Drugs reported as useful in the treatment of acute leukemia are listed in table 1. Included in this table are columns indicating the probable critical cellular determinants for action within the cell, where known, and the probable lethal site of action of the agent.

TABLE 1.—Drugs useful in the treatment of acute leukemia, the probable critical cellular determinants for action within the cell, and the probable lethal site of action of the agent

Compound	Cellular determinant	Site of lethal action
Prednisone		Cell-membrane integrity
Methotrexate	Cell-membrane uptake	Blocks uracil deoxyriboside ➤ thymidine
6-Mercaptopurine	Intracellular phosphorylation	Blocks purine biosynthesis
Cyclophosphamide	Intracellular binding	Blocks RNA and ?DNA synthesis
Vincristine	Cell-wall permeability	Blocks RNA and microtubules
Cytarabine	Intracellular phosphorylation	Blocks DNA polymerase

For methotrexate (MTX), incubation of isolated human blasts in a medium containing the tritiated drug permits an estimate of the amount transportable into the leukemic cell. This has been shown to correlate well with the capacity of such cells to respond to MTX, both in murine and human leukemia (text-fig. 1). MTX once taken up is active as such and should then inhibit the enzyme dihydrofolate reductase to a variable degree. This inhibition, in turn, results in a variable inhibition of the synthesis of thymidine from the usual endogenous precursor, deoxyuridine. These two effects can be assayed in human leukemic blasts sampled from the peripheral blood and bone marrow *(1)*.

With purine and pyrimidine bases or nucleosides, such as 6-mercaptopurine (6-MP) *(2)* and cytosine arabinoside (CA) *(3)*, phosphorylation must precede drug lethality. If a cell does not have the metabolic machinery to convert such an artificial drug analog of a normal precursor to a nucleotide, it cannot "self-destruct." By incubating the cells of interest with isotopically labeled drug, one can estimate the amount of activation by phosphorylation that has taken place (text-figs. 2, 3). This

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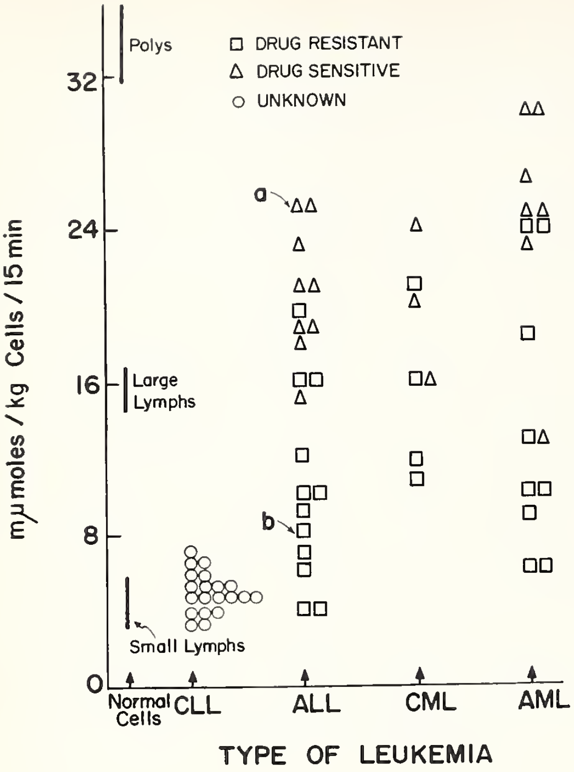
[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

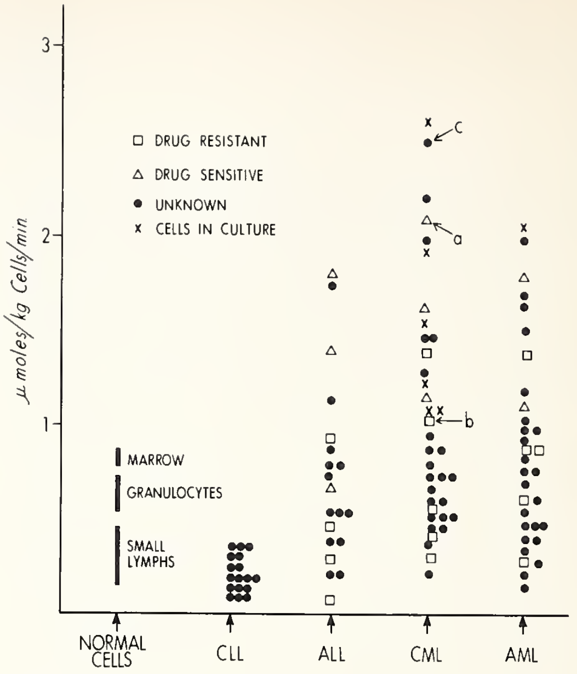
[3] Supported in part by Public Health Service contract PH69-39 and grant CA 11198 from the National Cancer Institute.



Text-figure 1.—Uptake of MTX after incubation for 15 minutes at 37°C in medium containing 0.2 μM drug. Sources of cells: CLL, chronic lymphocytic leukemia; ALL, acute lymphocytic leukemia; CML, chronic myelogenous leukemia; and AML, acute myelogenous leukemia. Data on normal cell types were obtained from at least 10 different preparations for each cell type. The range of results obtained is shown. Drug uptake was significantly different ($P<0.01$) in sensitive versus resistant AML and ALL cells, but not ($P = 0.2$) in the more limited number of CML cell preparations tested.

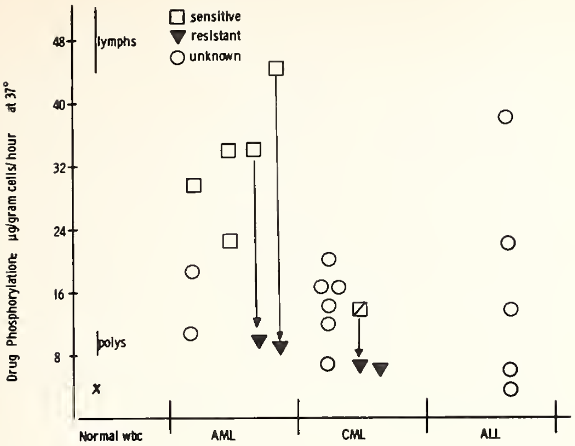
process is simplified by use of whole cells, since the nucleotide, when formed within the cell, cannot be transported back out by a mammalian cell wall. Hence the amount of isotope remaining within the cell following a short *in vitro* incubation of 15–30 minutes is a good index of the relative ability of a cell to activate the drug in question. Again, the inhibition of DNA synthesis by such blast cells can be measured as a "final common path" of antimetabolite drug action.

However, compounds such as cortisone, vincristine, and cyclophosphamide cannot be studied by the above method. They either are not available in isotopically labeled form or have no limitations to membrane transport, or have no known pathways of



Text-figure 2.—Conversion of 6-MP to nondiffusible metabolites during incubation for 15 minutes at 37°C in medium containing 0.1 mM drug. Sources of cells: CLL, chronic lymphocytic leukemia; ALL, acute lymphocytic leukemia; CML, chronic myelogenous leukemia; and AML, acute myelogenous leukemia. Data on normal cell types were obtained from at least 10 different preparations (4 marrow preparations). Cells were freshly isolated from blood except for × preparations which were obtained from samples of cells grown in spinner culture. Significant differences ($P<0.05$) in drug conversion were found between sensitive and resistant cell types. Analysis of variance revealed no other significant differences. Leukocyte samples *a* and *b* were obtained from a patient before and after, respectively, development of 6-MP resistance. Sample *c* was obtained from a patient successfully treated with radiation therapy. At a later time, the patient was found to be clinically responsive to 6-MP.

metabolic activation. Hence the potential capacity of these compounds to induce remissions after a month of treatment cannot be inferred from a 15-minute *in vitro* incubation. However, to be effective, these compounds must inhibit critical macromolecular biosynthesis, *e.g.*, DNA, after they are given in usual therapeutic doses. Patients in whom one of these drugs will, in 30 days' time, induce a remission might reasonably be expected to have significant DNA inhibition over the first 2–4 days of therapy. Such drug sensitivity by tumor cells also might reasonably be expected to occur



TEXT-FIGURE 3.—CA phosphorylation versus response in human leukemias. wbc, white blood cells; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; and ALL, acute lymphocytic leukemia.

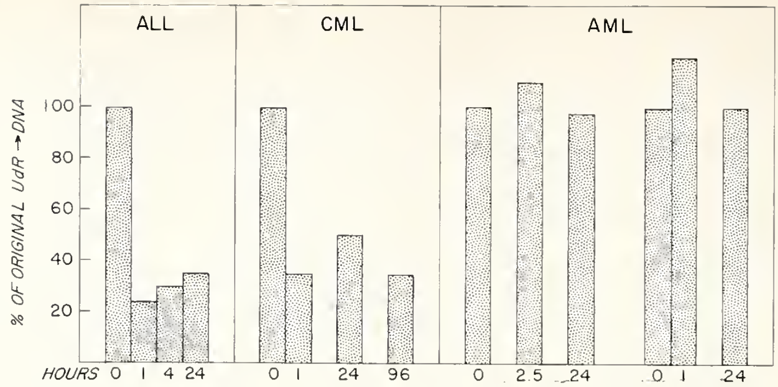
soon after first treatment and in time to prevent toxicity to normal tissues by causing discontinuation of agents which show little effect on tumor cells. Text-figure 4 shows the lack of inhibitory effect on DNA synthesis from deoxyuridine and thymidine of a single infusion of fluorouridine (FUR) in a series of drug-resistant patients with acute myelogenous leukemia. This drug was conceived of as a new agent because of theoretical considerations leading me to believe that it could bypass the biochemical block which results in the inactivity of 5-fluorouracil in humans. If single-pulse doses had not been effective in this type of preliminary experiment, one would not have gone to the trouble of making enough drug to undertake a full-scale clinical trial. Moreover, inhibition of DNA, such as is shown here for 50% of the patients, can be used to recruit the interest of clinicians in a new drug trial.

The inhibitory effect of vincristine on DNA synthesis in sensitive leukemic cells can be shown in the same fashion. Again, the method is to draw a pretreatment sample, inject a dose of the usual therapeutic size, and draw post-therapy samples at intervals thereafter. The maximum effect appears 24–48 hours after the drug is given. The ability of such a test to demonstrate the point in time at which maximal effect is seen indicates a second major benefit of this system, that of suggesting appropriate intervals for scheduling clinical drug doses. As opposed to a system of *in-vitro* testing, this treat-*in-vivo*–test-*in-vitro* system permits all the usual clinical therapeutic factors, such as blood levels, plasma binding, renal excretion, and hepatic activation or detoxification, to take the roles that they would in the actual clinical situation *(4)*.

RESULTS

A group of children with acute lymphocytic leukemia, most of whom had relapsed on previous therapy, were examined by the above techniques for drug metabolism and DNA effects of pulse doses given at usual therapeutic levels. Many of these children had very low counts or were small in size, and the amounts of bone marrow available for analysis were too small to permit examination of all drugs desired. Commonly, clinical reasons prevented repeated sampling, so that serial studies of drug effects on DNA metabolism could not be performed. These difficulties would be less formidable with our current smaller scale techniques and in a clinical setting modified so as to facilitate such research. Nevertheless, we could obtain samples of cells from patients known to the pediatricians to be resistant to some drugs. In most cases, we were not aware of the specific drugs to which the patients had developed clinical resistance. Hence these tests can be considered a "retrospective prediction." In table 2, we list the results of the tests on drugs specified by the clinicians as compared with the known clinical resistance, as made available to the laboratory at a later date. The correlation is gratifying from this small number of patients.

Many patients were studied before their going onto drug protocols. When the tests were done, we were unaware of what drugs or combinations of drugs were to be used. Sometimes, the results of tests, when indicative of a high level of drug handling by the blasts, contributed to the choice of agents for clinical use. It could not be otherwise at the time that we were developing the methodology. Pediatric oncologists are a skeptical group, justifiably unimpressed by the relevance of their work to another new test. Table 3 shows the results of these "prospective" predictive studies. Optimally, we would have a firm positive prediction upon very high uptake and retention in a labeled drug by the cells *in vitro,* followed by a drug-induced inhibition of 75% or more of the initial rate of DNA synthesis. In most instances, it was not possible to get all these



Text-figure 4.—Effect of fluorouridine on uracil deoxyriboside (UdR) incorporation into DNA in human leukemias. ALL, acute lymphocytic leukemia; CML, chronic myelogenous leukemia; and AML, acute myelogenous leukemia.

Table 2.—Acute lymphocytic leukemia: Retrospective biochemical prediction of response

Patient No.	Initials	Drug	Test	Prediction	Clinical response	Correlation[*]
1	D.Am.	CA	Uptake	—	—	+
2	D.Mi.	MTX	Uptake	—	—	+
3	M.Ho.	CA	Uptake	—	—	+
4	J.Su.	CA	Uptake	—	—	+

[*] Prediction for response or failure was followed by confirmation of this prediction after a clinical course of drug (+).

Table 3.—Acute lymphocytic leukemia: Prospective biochemical prediction of response

Patient No.	Initials	Drug(s)	Test(s)	Prediction[*]	Clinical response[†]	Correlation[‡]
5	M.No.	MTX	Uptake	+	C.R.	+
6	M.Jo.	MTX	Uptake	—	C.R.	—
7	R.Al.	MTX	Pulse	+	C.R.	+
			UdR[§]	+		
			dHfR[‖]	+		
8	M.Ch.	MTX	Pulse	+	C.R.	+
		(+ prednisone)	dHfR	+		
9	S.Gr.	MTX	Uptake	±	P.R.	+
			Pulse	±		
10	M.Gr.	MTX	Uptake	+	—	±
			UdR	—		
11	K.Gr.	MTX	Uptake	+	C.R.	+
12	D.McC.	MTX	Pulse	+	C.R.	+
13	A.Ka.	MTX	Uptake	+		
			UdR	—	C.R.	±
		(+ CA)	Uptake	—		
14	M.No.	CA	Uptake	—	—	+
15	P.Ua.	CA	Pulse	—	—	+
16	J.Su.	CA	Uptake	+	C.R.	+
17	R.Al.	6-MP	Pulse	—	—	+
18	C.Wa.	6-MP	Uptake	—	—	+
19	P.Zi.	FUR	Pulse	—	—	+

[*] Prediction: failure of drug treatment (—); likelihood of success (+); possible partial remission (±).

[†] C.R.: complete response as defined by the acute leukemia group B. P.R.: partial response as defined by the acute leukemia group B.

[‡] Correlation: prediction for response or failure was followed by confirmation of this prediction after a clinical course of drug (+).

[§] UdR: inhibition of uracil deoxyriboside incorporation into DNA by leukemic cells after treatment of patient with test drug.

[‖] dHfR: inhibition of dihydrofolate reductase activity in leukemic cells after treatment of patient with test dose of MTX.

data. We resorted to making firm negative predictions if a specific drug was not taken up or phosphorylated by the whole cell or if a pulse dose of usual therapeutic size given to the patient produced no discernible inhibition of DNA (as indicated above) in marrows essentially replaced by blasts.

When the uptake of DNA inhibition was in the middle range or there was no opportunity to follow uptake with a pulse-dose DNA inhibition study or the clinicians proposed to add a first drug with some uptake to a second drug which we had not studied, we could only predict "possible response." This prediction of "permissive" action appears as a ± in the "Prediction" column. Table 3 again shows correlations which suggest enough accuracy to justify a controlled trial. This would include more careful examination of each patient's cells, several "DNA pulses," and careful control of therapy, so that, for a fixed number of patients, treatment would be assigned on the basis of best clinical practice in the absence of any knowledge of the laboratory results. At the end of the study, a valid comparison could be made of the predictions made at the beginning of study with the results of chemotherapy.

In acute myelogenous leukemia of adults, there is a much greater need for this type of predictive capacity, because there is a larger number of less effective drugs for acute myelogenous leukemia than for acute lymphatic leukemia and the course

TABLE 4.—Acute myelogenous leukemia: Responders

Patient initial	Uptake and retention		DNA inhibition			Clinical treatment	
	Drug	Predictive value*	Drug	Uracil deoxyriboside, predictive value*	Thymidine, predictive value*	Regimen used	Response achieved
E.T.	MTX	+	VAMP†	+	+	VAMP	Complete (slow)
	6-MP	+					
J.C.	CA	+	CA	+	+	CA	Complete
R.W.	MTX	+	CA	+	+	MTX + CA	Toxic
	CA	±					
M.S.	CA	+	CA	+	±	CA	Partial
A.K.	*a*) MTX	+	VAMP				Partial
	6-MP	—		±	±	VAMP	
	b) CA	+	CA + VAMP	+	±	CA + VAMP	Partial
	c) Daunomycin		Daunomycin	+	+	Daunomycin + CA + VAMP	Complete
S.T.	MTX	+					
	CA	±				VAMP	Complete
	6-MP	±					
R.S.	MTX	±				VAMP	Partial
	CA	±					
	6-MP	—					
K.F.	MTX	±				CA	
	6-MP	±				6-MP	Complete (slow)
	CA	+				VCR	
	FUR	+					
F.L.	FUR	+					
	CA	±					
	MTX	±					
	6-MP	±				CA, cyclophosphamide, and MTX	Partial
E.S.	MTX	+					
	CA	+					
	FUR	+					
	6-MP	±	Prednisone	—	—	CA, MTX, and	
			Vincristine	±	±	vincristine	Complete

*Prediction: failure of drug treatment (—); likelihood of success (+); possible partial remission (±).

†VAMP: combination of vincristine, MTX, 6-MP, and prednisone.

of acute myelogenous leukemia is very rapid and lethal. If a drug is successful, the patient may survive, and, if it fails, there usually will not be another chance to select an effective drug. Another reason for the greater need for a predictive test in human leukemia is the current interest in multiple drug combinations. If an ineffective drug can be identified and thus omitted from the proposed drug regimen, it will avoid unnecessary drug toxicity to the host. Also, if most or all of the proposed drugs are not activated by, retained by, nor cause DNA inhibition in the blasts—as well might be expected to occur in the 60% of patients with acute myelogenous leukemia who currently do not respond to any traditional drug combination—these patients could, quite justifiably, be started immediately on new and investigational drugs.

Table 4 shows the results of clinical therapy in patients in whom we predicted failure of therapy with the drugs studied. In general, the correlation is good, although often there was only one drug tested and multiple drugs were actually given to the patients. As in patients with acute lymphocytic leukemia, there was often insufficient opportunity to confirm the uptake suggestions by administering test doses of drugs to the patients. Nevertheless, in most instances, no remissions or poor ones were achieved in patients who had poor pharmacologic predictions.

Table 5, on the other hand, shows the patients whose laboratory tests suggested that they would be sensitive to some degree to the drugs tested. There were few definitely positive predictions, a reflection of the resistance of acute myelogenous leukemia to drugs which are commonly much more effective in acute lymphocytic leukemia. Most predictions were of the "permissive positive" type, based on lack of data or on intermediate levels of drug uptake or DNA inhibition. The overall correlations are reasonably good. We feel that again the data are sufficiently promising to undertake a more controlled trial of the predictive system.

Table 5.—Acute myelogenous leukemia: Nonresponders

	Uptake and retention		DNA inhibition			Clinical treatment	
Patient initial	Drug	Predictive value*	Drug	Uracil deoxyriboside, predictive value*	Thymidine, predictive value*	Regimen used	Response achieved
R.D.	CA	—	CA	—	—	CA	None
R.W.	6-MP	—	6-MP	—	—	6-MP	None
D.J.	CA	—				CA	None
A.W.	MTX	+				VAMP†	None
	6-MP	—				VAMP + CA	None
	CA	—					
R.D.	MTX	+	MTX	+	—	VAMP	None
	6-MP	—	6-MP	±	—		
G.P.	CA	—	CA	±	±	CA	None
J.H.	CA	+	CA	±	±	CA + MT	Poor partial
	FUR	+	FUR	±	±	Vincristine	
	MTX	±	MTX	—	—		
	6-MP	—					
			Vincristine	—	—		
			Cyclophosphamide	+	+		
M.Mc.	MTX	+	MTX	—	—		
	FUR	+					
	CA	±					
	6-MP	—					
			Vincristine	±	±	CA + vincristine	Poor partial

*Prediction: failure of drug treatment (—); likelihood of success (+); possible partial remission (±).

†VAMP: combination of vincristine, MTX, 6-MP, and prednisone.

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DNA Synthesis in Leukemic Cells Under the Action of Cytotoxic Agents *In Vitro* and *In Vivo*[1, 2]

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MOST OF the cytotoxic drugs used in the treatment of acute leukemia act by interfering with nucleic acid synthesis. Antimetabolites of pyrimidine metabolism, such as folic acid antagonists and cytosine arabinoside (Ara-c), are potent inhibitors of DNA synthesis. The most important enzymatic reactions essential for DNA synthesis and the target sites of methotrexate (MTX), 5-fluorouracil (5-FU), and Ara-c are demonstrated in text-figure 1. MTX and 5-FU interfere with the *de novo* synthesis of thymidylate. MTX and 5-FU inhibit the dihydrofolate reductase *(1–4)* and the thymidylate synthetase *(5–7)*, respectively. Ara-c inhibits DNA polymerase *(8)* and cytidine-diphosphate reductase *(9)* which leads to the deoxy derivative. Phosphorylation of this drug to the triphosphate and incorporation into DNA are discussed *(10)*.

Other important drugs used in the treatment of acute leukemia, such as daunomycin, vincristine, 6-mercaptopurine (6-MP), and corticosteroids, are not listed in this scheme. Daunomycin and adriamycin supposedly interfere with DNA-dependent RNA polymerase, and 6-MP with purine nucleotide synthesis. The mechanism of action of vinca alkaloids is still obscure. Nucleic acid synthesis and the mitotic phase are discussed as target sites. Corticosteroids are effective only in lymphoblastic leukemia by cytolysis of the lymphatic cells and probably by interference with DNA synthesis.

As we reported previously, the thymidine (TDR) kinase increases if the dihydrofolate reductase is inhibited by MTX *(11)*. This increase occurs also during treatment with Ara-c *(12)*. These *in vivo* observations of patients under therapy with one of the two drugs agree with results of other investigators on cell cultures [Morse and Potter *(13)*, Rubini *(14)*, Wuest *(15)*, and Kit and co-workers *(16)*].

The increase of TDR kinase activity under these circumstances can be explained by: 1) synchronization of cells in the DNA synthesis phase and 2) the control function of thymidine triphosphate (TTP), which is formed by phosphorylation of the thymidine monophosphate (TMP) *(17–21)*. TTP inhibits TDR kinase. The increased activity of TDR kinase under the action of these drugs can therefore be explained by MTX-induced inhibition of the *de novo* synthesis of thymine nucleotides or by reduced formation of deoxyuridine monophosphate (dUMP) from deoxycytidine monophosphate (dCMP) under the action of Ara-c (text-fig. 1). The TDR kinase can compensate for the cytotoxic effect of MTX *(11, 21)* but not for that of Ara-c *(22)*. Since this antimetabolite inhibits primarily the reduction of cytidine diphosphate (CDP) to deoxycytidine diphosphate (dCDP) and, therefore, the formation of deoxycytidine triphosphate (dCTP), this latter compound is not available for reproduction of DNA in the polymerase reaction *(9)*. These two different effects of increased TDR kinase activity on DNA synthesis are confirmed by incorporation studies with ^{3}H-labeled thymidine (^{3}H-TDR) and ^{3}H-labeled deoxyuridine (^{3}H-dUR) as specific DNA precursors of the salvage and *de novo* pathway.

Text-figure 1 demonstrates that not only TDR kinase but also other enzymes necessary for DNA synthesis, such as deoxyuridine (dUR) kinase and dCMP deaminase *(20)*, are controlled through negative feedback inhibition by TTP. Therefore, we can expect a decreased utilization of ^{3}H-TDR if an excess of unlabeled dUR is added to a cell culture and, conversely, a repression of ^{3}H-dUR incorporation if unlabeled TDR is available in excess (text-fig. 2). The experiments shown here and in text-figures 3 through 11 were done with cultures of leukemic cells incubated for 4 hours. The incorporation of DNA precursors was measured in the trichloroacetic-acid-insoluble fraction on Millipore filters with a Packard Liquid Scintillation Spectrometer. Text-figure 2 demonstrates the inhibition of ^{3}H-TDR incorporation into DNA by the addition of unlabeled dUR and the inhibition

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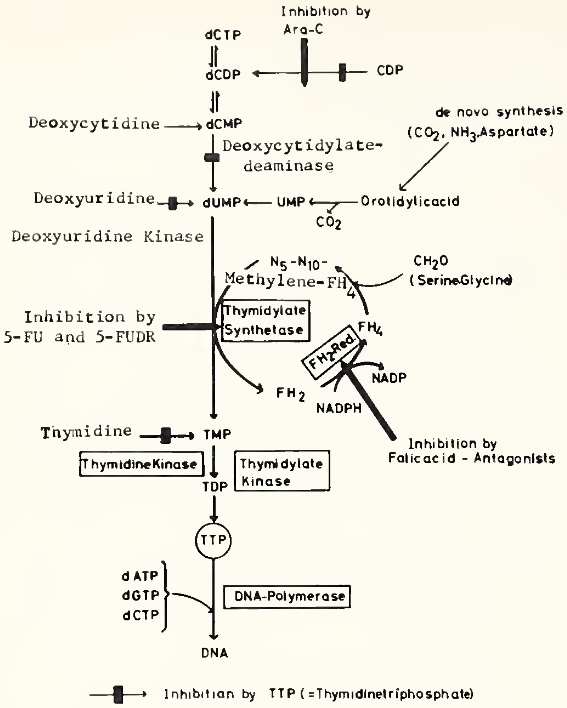
[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

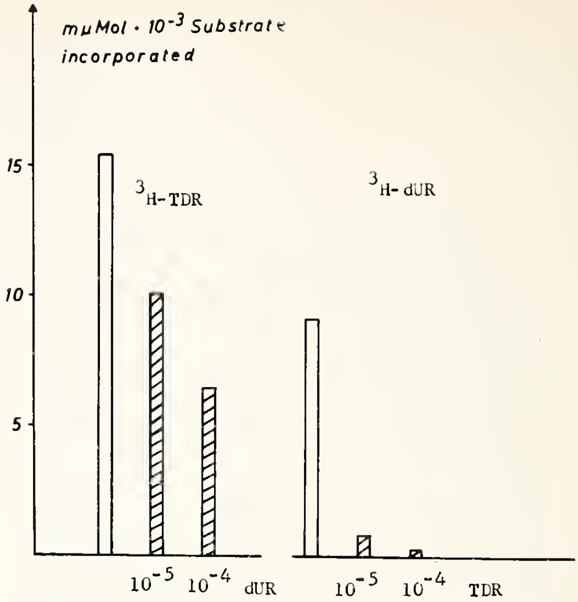


Text-figure 1.—TDR nucleotide synthesis, its regulation by thymidine triphosphate (TTP) and the sites of action of antimetabolites. dCMP = deoxycytidine monophosphate; dCDP = deoxycytidine diphosphate; dCTP = deoxycytidine triphosphate; UMP = uridine monophosphate; dUMP = deoxyuridine monophosphate; TMP = thymidine monophosphate; TDP = thymidine diphosphate; dATP = deoxyadenosine triphosphate; dGTP = deoxyguanosine triphosphate; Ara-c = cytosine arabinoside; 5-FU = 5-fluorouracil; 5-FUDR = 5-fluorodeoxyuridine; FH_2 = dihydrofolic acid; and FH_4 = tetrahydrofolic acid.

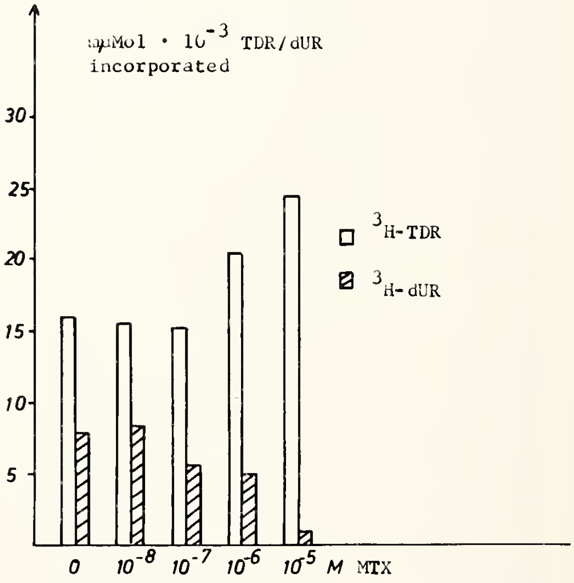
of ^{3}H-dUR incorporation by the addition of unlabeled TDR to the cell culture.

Methotrexate

As a consequence of this interrelation between dUR and TDR utilization for DNA synthesis, the incorporation of ^{3}H-TDR is increased when the *de novo* synthesis of TMP—and, therefore, the utilization of dUR—is inhibited by the folic acid antagonist, MTX. Text-figure 3 illustrates the inhibition of ^{3}H-dUR incorporation in the acid-insoluble fraction by an MTX concentration of from 10^{-7}–10^{-5} M in the culture medium and a higher incorporation of ^{3}H-TDR with increased concentrations of the drug. In accordance with these results were the experiments shown in text-figure 4. The inhibition of ^{3}H-TDR incorporation by an excess of unlabeled dUR was reversible if MTX was added. Adding



Text-figure 2.—Inhibition of ^{3}H-TDR incorporation by dUR and of ^{3}H-dUR incorporation by TDR. Added to the cell culture was 1 mμmole of ^{3}H-labeled precursors. Incubated for 4 hours were 1.46 × 10^6 cells. Patient, Hin., was a 44-year-old male with acute erythroleukemia.



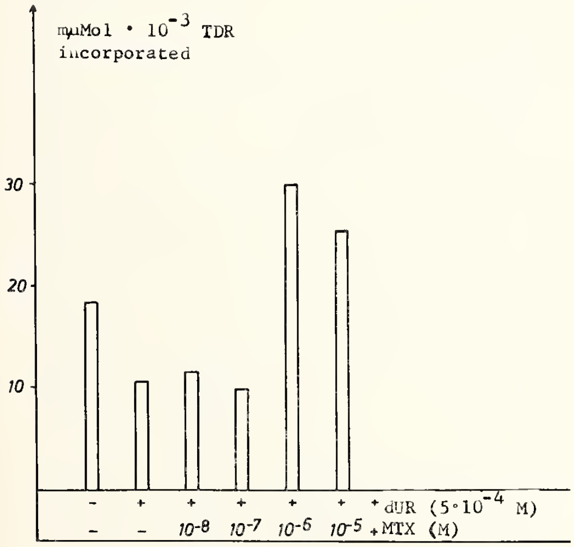
Text-figure 3.—Effect of MTX on ^{3}H-TDR and ^{3}H-dUR incorporation. Incubated for 4 hours were 2.94 × 10^6 cells. Patient Se. was a 25-year-old male with acute blast crisis in chronic myelocytic leukemia.

the folic acid antagonist to the dUR-containing medium even increased the incorporation rates of ^{3}H-TDR compared to those of control cells incubated without dUR.

From these results it can be concluded that a rise in TDR kinase activity under the influence of MTX—which I reported previously—enhances the utilization of TDR for DNA synthesis and, therefore, compensates for the inhibition of dihydrofolate reductase in resistant cells.

Our own results *(22)*, describing a rather complex situation with regard to the sensitivity or resistance of cells to MTX, are as follows. Sensitivity of the cells can be assumed if: 1) The dihydrofolate reductase is inhibited completely and immediately after a single intravenous injection of 20 mg MTX; 2) the inhibition lasts more than 3 days—a time which is related to the cell cycle; 3) the intracellular concentration of the drug is high enough to inhibit newly induced dihydrofolate reductase; and 4) the compensation mechanism caused by the rise in TDR kinase activity is disturbed.

Roberts *et al. (23, 24)* recently showed that induction of thymidylate synthetase may also have a function in the development of resistance of leukemic cells to MTX.



Text-figure 4.—Effect of dUR (5×10^{-4} M) and MTX on incorporation of ^{3}H-TDR. Incubated for 4 hours were 3.34×10^6 cells. Patient Se. was a 25-year-old male with acute blast crisis in chronic myelocytic leukemia.

Cytosine Arabinoside

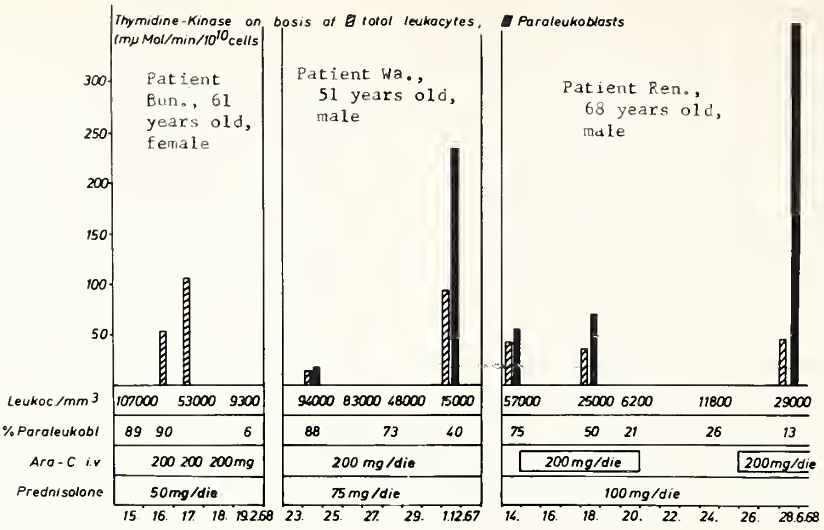
As I mentioned, therapy with Ara-c also frequently increased TDR kinase activity (text-fig. 5). But in contrast to MTX, as shown by *in vitro* experiments, Ara-c inhibited both ^{3}H-dUR and ^{3}H-TDR incorporation into DNA (text-fig. 6). This was explained by the lack of dCTP which is necessary as a substrate for DNA polymerase. The reason for the increased TDR kinase activity may have been a loss of feedback inhibition by TTP and also a partial synchronization of the cells during the DNA synthesis phase. As can be seen in text-figure 7, under treatment, the incorporation rates of dUR and TDR were significantly reduced 1 hour after intravenous injection of 1.5 mg Ara-c/kg. But this inhibition lasted only a few hours, probably because the drug was rapidly inactivated by deamination *(25)*. Therefore, the drug had to be injected frequently to achieve a remission. (On the other hand, to prevent the normal proliferating cells from being destroyed, the drug should not be administered too frequently. For this reason, continuous infusion is not favored.) On the basis of these theoretical considerations, we obtained encouraging results by injecting the drug in divided doses of 1.5 mg/kg, 2 times daily.

That the increased activity of TDR kinase under treatment with Ara-c is at least partially caused by interference with enzymatic regulation of DNA synthesis is shown in text-figure 8. *In vivo,* the enzyme activity was compared with the TDR-labeling index. The experiments were performed in collaboration with Doctor Hartje of our clinic. During this patient's illness with acute myeloblastic leukemia, the TDR kinase was extremely elevated, even when the number of tritium-labeled cells and blast cells were already significantly reduced after treatment with Ara-c.

The experiments described can be applied to other cytotoxic drugs and can be used as a predictive test system in the treatment of acute leukemia.

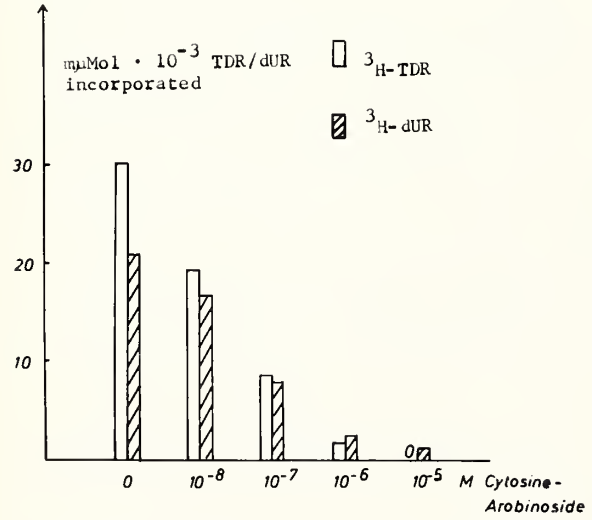
Daunomycin

The antibiotic, daunomycin, complexes with DNA and inhibits DNA-dependent RNA polymerase *(26)*. *In vitro* the incorporation rates of ^{3}H-dUR and of ^{3}H-TDR into DNA and of ^{3}H-uridine (^{3}H-UR) into RNA were reduced significantly by concentrations between 10^{-6} and 10^{-5} M daunomycin in the culture medium (text-fig. 9).



TEXT-FIGURE 5.—TDR kinase in leukemic cells under treatment with Ara-c.

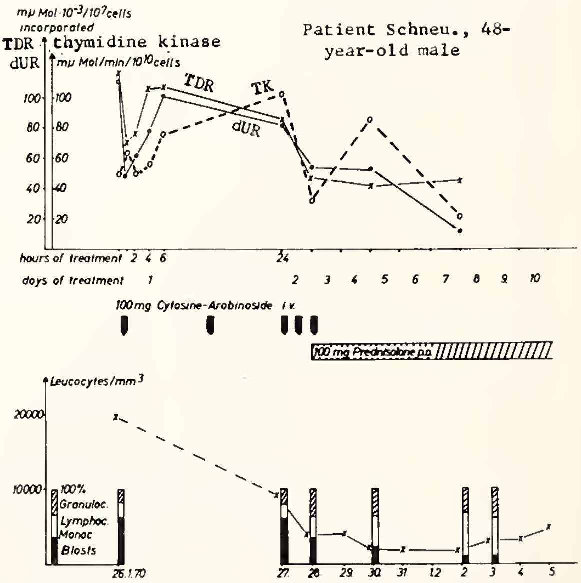
An injection of 1 mg/kg daunomycin *in vivo* had the same effect on DNA and RNA synthesis in sensitive leukemic cells, which could be observed after 24 hours (text-fig. 10). The activity of TDR kinase was also decreased during the first 24 hours. It is important to notice that incorporation rates of DNA and RNA precursors were reduced before morphologic changes in the differential blood count could be observed.



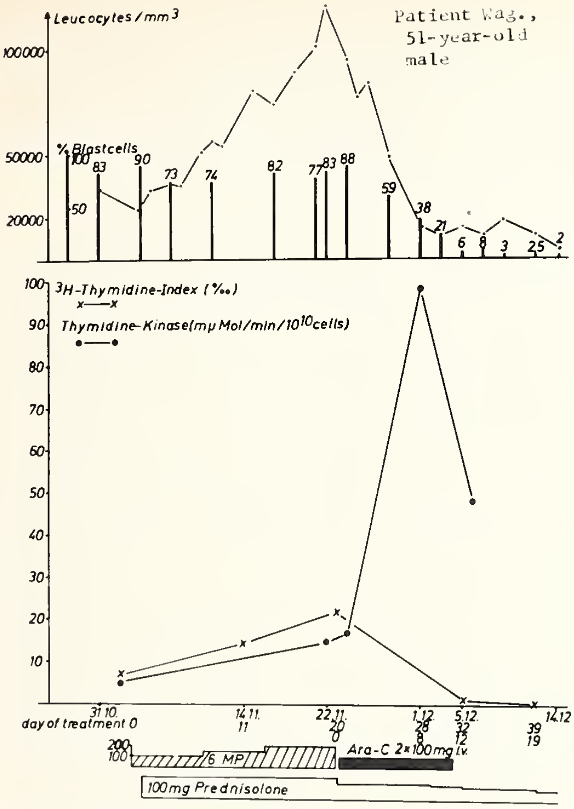
TEXT-FIGURE 6.—Effect of Ara-c on TDR and dUR incorporation. Incubated for 4 hours were 1.22 × 10^6 cells (bone marrow). Patient Ka. was a 53-year-old female with acute leukemia.

Corticosteroids

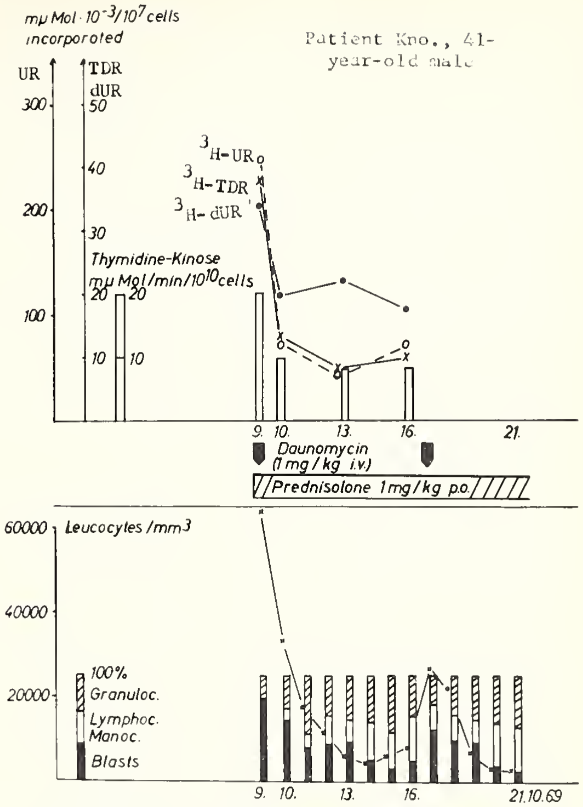
Corticosteroids are mainly effective in the treatment of acute lymphoblastic leukemia. Lysis of lymphatic cells and reduction of DNA and RNA synthesis in lymphoid cells are possible mechanisms of action proposed by different investigators *(27)*. However, the problem has not yet been satisfactorily



TEXT-FIGURE 7.—TDR and dUR incorporation, TDR kinase, leukocytes, and differential count of a patient with acute leukemia during treatment with Ara-c.

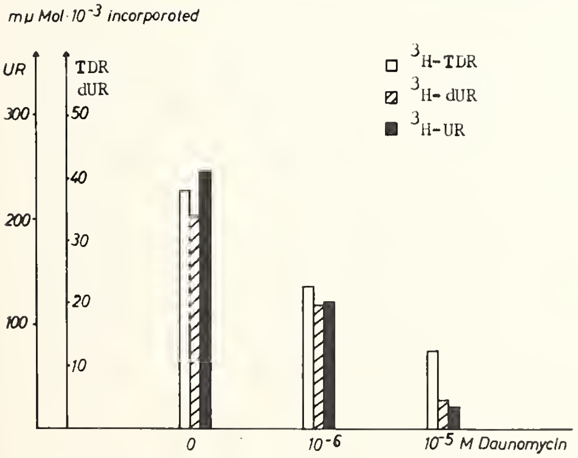


Text-figure 8.—^{3}H-TDR-labeling index, TDR kinase, leukocytes, and blast cells in the differential count of a patient with acute myeloblastic leukemia treated with 6-MP, Ara-c, and prednisolone.



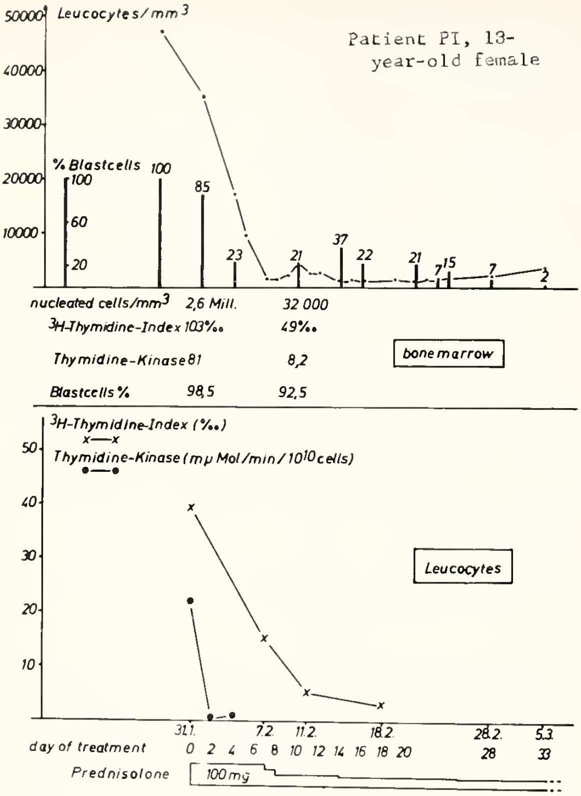
Text-figure 10.—Incorporation of DNA and RNA precursors, TDR kinase, leukocytes, and differential count of a patient with acute myeloblastic leukemia treated with daunomycin and prednisolone.

solved. *In vitro* no significant effect on DNA and RNA synthesis could be detected, nor were enzymatic reactions in the cytolysates of lymphoblasts affected *(27)*. *In vivo,* however, in all patients with lymphoblastic leukemia successfully treated with prednisolone, a rapid fall in TDR kinase activity could be observed as the earliest event, 24–48 hours after treatment with 1.5 mg/kg daily was begun (text-fig. 11). Incorporation rates of labeled TDR and dUR also decreased, but not constantly. The reason for the rapid fall in TDR kinase activity at the beginning of prednisolone treatment may have been a repression of enzyme synthesis. This effect seems to be limited to lymphoid cells, because, in other leukemic, nonlymphoblastic cells, a similar decrease in TDR kinase activity has never been observed. As a consequence of these findings, only lymphoblastic leukemia should be treated with corticosteroids in high doses.



Text-figure 9.—Effect of daunomycin on incorporation of ^{3}H-labeled DNA and RNA precursors. Patient Kno. was a 41-year-old male with acute myeloblastic leukemia.

Table 1 summarizes the correlation between *in vitro* and *in vivo* experiments in our laboratory for predicting the response of patients with acute leukemia and gives the clinical results obtained by



TEXT-FIGURE 11.—^{3}H-TDR-labeling index, TDR kinase, leukocytes, and blast cells in the differential count of a patient with acute lymphoblastic leukemia treated with prednisolone.

therapy with MTX, Ara-c, daunomycin, and prednisolone.

An important factor for the sensitivity of leukemic cells against any cytotoxic drug is the concentration of the metabolically active drug within the cells. Hall, Kessel, Bertino, and other investigators have demonstrated this for MTX and Ara-c. Changes of patterns of nucleic acid synthesis can occur only if the concentration of the metabolically active drug in the target cells is high enough. Therefore, these experiments seem to offer a suitable test system to predict the response of the leukemic process to a specific treatment and to improve methods of timing and of dosage of specifically acting drugs.

SUMMARY

In leukemic cell cultures incubated for 4 hours, the incorporation of tritium-labeled TDR and dUR into DNA was determined. The activity of the TDR kinase was also determined in the cytolysates of the leukemic cells.

1) Unlabeled TDR inhibited the incorporation of ^{3}H-dUR and, reciprocally, unlabeled dUR inhibited the incorporation of ^{3}H-TDR. These relations were explained by feedback inhibition of dUR kinase and TDR kinase by TTP.

2) MTX inhibited the incorporation of dUR. This was based on the blockade of the *de novo* synthesis of thymine methyl groups. MTX increased the utilization of TDR for DNA synthesis. This was explained by a reduction in the negative feedback control of TDR kinase by TTP.

3) Ara-c decreased incorporation of both dUR and TDR. It inhibited the synthesis of TTP and dCTP—both substrates of DNA polymerase. Therefore, despite the increased activity of TDR kinase, TDR cannot be utilized for DNA synthesis.

4) A single dose of daunomycin caused, in sensitive leukemic cells, a significant decrease in incorporation of labeled precursors of DNA and RNA and, also, a fall in the TDR kinase activity during the first 24 hours.

5) Corticosteroids were only effective in lymphoblastic leukemias. A rapid fall in TDR kinase activity during treatment with prednisolone was the first sign of sensitivity observed.

TABLE 1.—Results of biochemical prediction tests in treatment of acute leukemia

	Number of tests		Therapeutic effects		
Drug	Positive	Negative	Complete or partial remission	Only decrease in leukocytes	No effect
Amethopterin (MTX)	6		4	2	
	2(+)			1	1
		5		1	4
Ara-c (AC 1075 "Mack")	14		10	3	1
		6	1	1	4
Daunomycin (daunoblastine)	6		3	3	
Prednisolone (hostacortine)	9		9		

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Anticancer Effects of Propanals: Comparison Between Human and Animal Tumor Systems[1, 2, 3]

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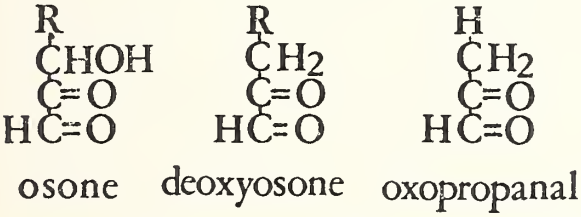
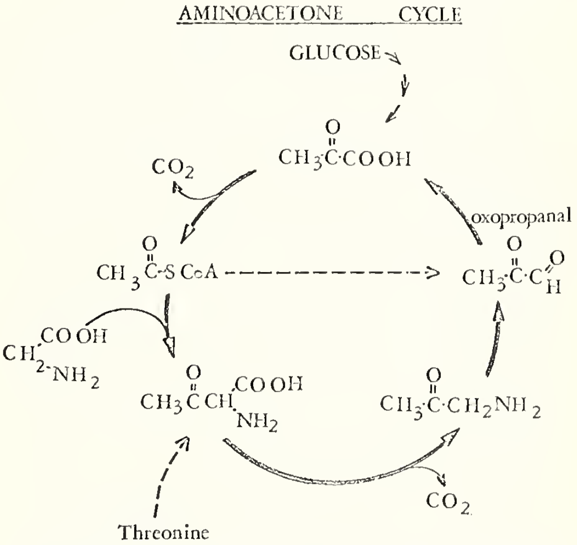
IN 1932, Bruno Kisch described the ability of 2-oxopropanal (2-OP) to inhibit the respiration of a number of tissues, including a tumor *(1)*. 2-OP is the lowest member of the deoxyosone series of sugars (text-fig. 1); it is a deoxytriosone. This simple sugar is a normal metabolite which appears to be formed either by threonine oxidation or glycine-acetyl coenzyme-A condensation via aminoacetone (text-fig. 2) *(2)* and perhaps by acetoacetate decarboxylation *(3)*.



Text-figure 1.—Structural features of osones, deoxyosones, and the cancer growth-inhibiting metabolite, 2-OP. 2-OP is a deoxytriosone.



Text-figure 2.—The aminoacetone cycle. Formation and further metabolism of 2-OP as they appear to occur in mammals [modified from Elliott's *(2)* bacterial cycle to exclude D-lactic acid metabolism which does not occur in mammals]. Glucose is metabolized to pyruvate which forms acetyl-CoA. Acetyl-CoA condenses with glycine to form amino oxobutyrate (also formed by threonine oxidation). Amino oxobutyrate decarboxylates to form aminoacetone, which is then deaminated to form 2-OP. The latter in mammals can be directly oxidized to form pyruvate.

After the observation that 2-OP had antiviral activity *(4)*, several reports described the antitumor properties of certain alkylaldoketones *(5, 6)*. In 1966 and 1967, Szent-Györgyi and his colleagues, after further elucidating the inhibition of bacterial cell growth by deoxyosones first studied by Yoder and Torgeson *(7)*, speculated on the possible involvement of osones in cancer or the control of cell division *(8, 9)*. In 1965, on the basis of these and other observations, Apple and Greenberg systematically investigated the effects of propanals and osones on a number of animal tumor systems *(10–14)*. First studying 2-OP, they observed 90–99% inhibition of the growth of Ehrlich ascites carcinoma, L1210 and L4946 leukemia, TA_3 mammary carcinoma, 6C3HED lymphosarcoma, and Sarcoma 180 in mice *(11)*. A single dose of 2-OP significantly inhibited advanced leukemia and produced indefinitely prolonged survival of mice with lymphosarcoma and carcinoma *(11, 12)*. These results were achieved at daily dose levels of about 80 mg/kg or with a single dose regimen of 225 mg/kg, which approximated 70–80% of the maximum tolerated dose (defined as that dose producing no deaths or loss of body weight $>15\%$ within 2 weeks). A series of oxyderivatives of ethane, propane, and butane with carbohydrate-like structure were studied, and these investigators concluded that com-

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[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

[3] Supported by Public Health Service research grants CA 11067 and CA 07723 from the National Cancer Institute.

pounds with an aldoketone or hydroxyaldehyde structure had antineoplastic activity. Detailed studies of the antitumor efficacy in animal model systems were reported for 2-OP and 2,3-dihydroxypropanal (DHP) *(11,12)*.

As a prelude to studies of these compounds in patients with cancer, it was of interest to compare their activity *in vitro* against animal tumor cells and normal and malignant human leukocytes.

MATERIALS AND METHODS

Leukocytes were obtained from normal human subjects and patients with hematologic malignancies (usually before drug therapy) or from $B_6D_2F_1$ mice carrying transplantable lymphocytic leukemia, and were prepared for cultivation *in vitro* by previously described methods *(15)*.

The measurement of the incorporation of radioactive thymidine into DNA, or uridine into RNA, and of labeled amino acids into protein has been described previously *(15,16)*, as has the measurement of $^{14}CO_2$ production from ^{14}C-labeled carbohydrates *(17)*.

Cellular respiration was measured with a Clark electrode and a Gilson model KM oxygraph.

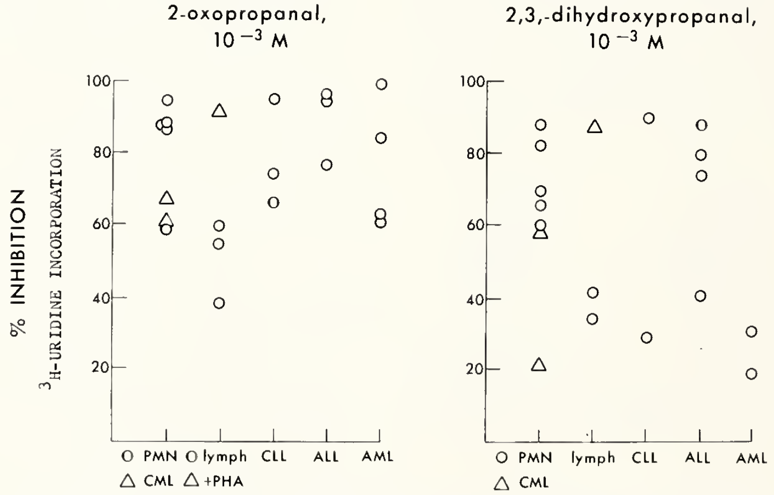
P388 lymphoid leukemia was obtained from I. Wodinsky of A. D. Little Company and maintained in ascites form in $B_6D_2F_1$ mice. Before *in vitro* studies, mouse leukemic cells were washed by centrifugation in a balanced salt solution supplemented with glucose and then resuspended in that solution.

RESULTS

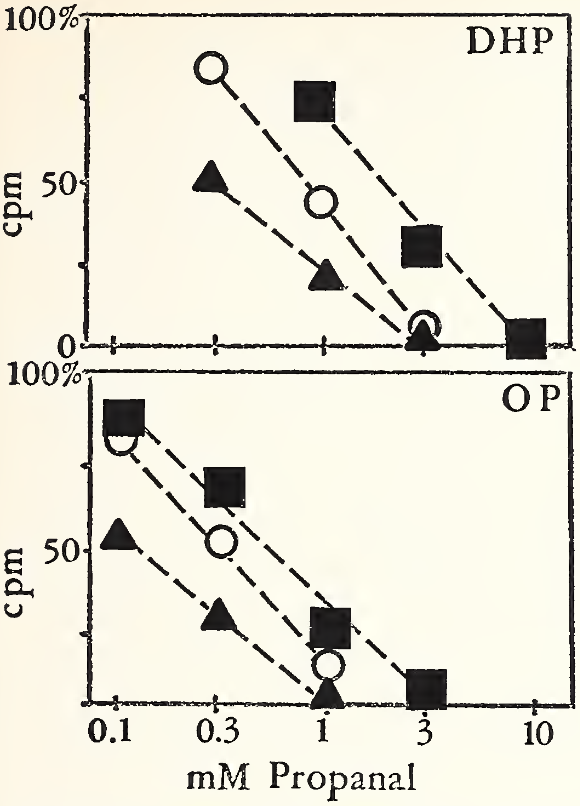
Nucleic Acid and Protein Synthesis

2-OP and DHP exerted dose-dependent effects on the incorporation of ^{3}H-uridine into the RNA of sensitive human leukocyte populations. Maximal effect was achieved at 10^{-3} M, and little effect was evident at concentrations below 10^{-4} M. As shown in text-figure 3, the human cells' incorporation of ^{3}H-uridine by normal human neutrophils and malignant, morphologically mature and immature lymphocytes, granulocytes, and plasma cells was sensitive to the effects of 2-OP and DHP. In contrast, normal mature human lymphocytes were less sensitive. However, when these normal lymphocytes were induced to undergo "blastogenic" transformation by phytohemagglutinin, they became sensitive (text-fig. 3).

The inhibition of murine leukemic cell protein and nucleic acid synthesis by 2-OP and DHP was linearly dependent on the logarithm of the dose over a wide range of propanal concentration (text-fig. 4) (4–6 hours' exposure). At each dose, the degree of inhibition was protein > RNA > DNA.



Text-figure 3.—Effect of 2-OP and DHP on the incorporation of ^{3}H-uridine into RNA of human leukocytes. ^{3}H-uridine was added 4 hours after addition of drug or saline (control), and incorporation of radioactivity into RNA was measured 18 hours later. PMN = normal granulocytes; CML = granulocytes from patients with chronic granulocytic leukemia; lymph = normal lymphocytes; PHA = phytohemagglutinin-stimulated lymphocytes; CLL = chronic lymphocytic leukemia; ALL = acute lymphoblastic leukemia; and AML = acute myelocytic leukemia.



TEXT-FIGURE 4.—Effect of 2 propanals on DNA, RNA, and protein biosynthesis by murine leukemic lymphocytes. Murine P388 lymphocytic leukemia cells were collected *(11)* during exponential growth phase *in vivo* and suspended in Eagle's minimal essential medium (Spinner, with glutamine). ^{3}H-thymidine incorporation (■), ^{14}C-uridine incorporation (O), or ^{3}H-leucine (▲) incorporation in trichloroacetic acid-precipitable polymers was used as the indicator of DNA, RNA, and protein biosynthesis, respectively. Cells were exposed to DHP or 2-OP for 6 hours, the last 4 hours including the indicated radioactive metabolite. The 100% values were all ~ 4000 cpm (liquid scintillation). Propanal concentrations are shown on a logarithmic scale.

Inhibition of human leukocyte nucleic acid and protein synthesis by 2-OP was maximal at the earliest period that could be conveniently measured after addition of drug (30–90 minutes) (text-fig. 5), and was achieved without loss of cell viability (trypan blue exclusion). Only after 18–24 hours' exposure to 2-OP *in vitro* did human leukocytes demonstrate morphologic evidence of cytolysis. Inhibition of incorporation of labeled nucleosides and amino acids reflected impaired synthesis of macromolecules but not diminished entry of labeled compounds into acid-soluble cellular pools. Murine leukemic cells exposed 7 hours to inhibitory concentrations of 2-OP were 90–95% viable.

Respiration and Carbohydrate Metabolism

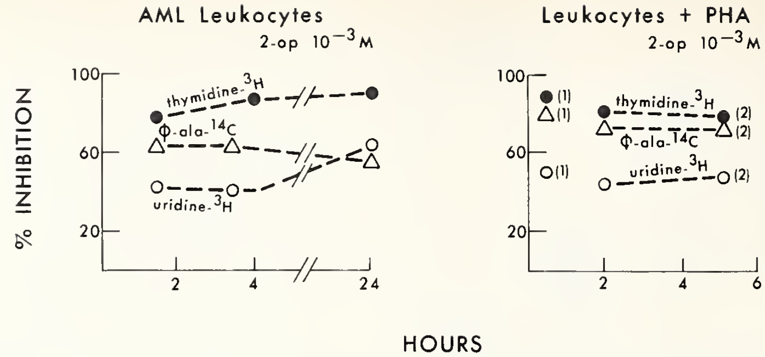
Oxygen consumption by a variety of normal and malignant human leukocytes in Eagle's minimal essential medium with fetal calf serum was unaffected by 10^{-3} M 2-OP for at least 5 hours, during which time profound changes in carbohydrate metabolism, nucleic acid synthesis, and protein synthesis were evident. In contrast, L1210 murine lymphocytic leukemic cells' oxygen consumption in glucose-isotonic solution was rapidly inhibited *(10)*.

The effect of 10^{-3} M 2-OP on the formation of $^{14}CO_2$ from a variety of labeled carbohydrates was studied over periods of 1–4 hours (table 1). 2-OP apparently had little effect on $^{14}CO_2$ generation from ^{14}C-2-pyruvate and reproducibly enhanced $^{14}CO_2$ production from glucose uniformly labeled or labeled in the C-1 or C-6 positions with both human and murine leukemic cells.

DISCUSSION

Although our original observations of *in vivo* activity have been confirmed and extended by others *(18)*, the precise mechanism of antitumor activity of 2-OP and other aldoketones is not known. 2-OP is known to inhibit succinic dehydrogenase *in vitro (19)* but can also be demonstrated to bind to DNA in cell-free systems *(20)*. Clearly the compound can exert cytotoxic effects in the absence of DNA-dependent DNA synthesis, as evidenced by its effects on mature neutrophils (an end cell incapable of DNA synthesis). Recent observations by Apple and Haskell, showing that 2-OP under some conditions can inhibit RNA-dependent DNA synthesis, may help to partially explain its antitumor activity *(21)*. The disparate effects of 2-OP on "resting" peripheral lymphocytes and "blastogenically" transformed lymphocytes are intriguing. Profound alterations of both carbohydrate and nucleic acid metabolism have been documented for lymphocytes undergoing transformation *(22)*.

There appear to be some differences between the *in vitro* effects of 2-OP on malignant human leukocytes and malignant mouse cells. Striking sensitivity of respiration and glucose oxidation of the L1210, but not the human leukemic, cell to 2-OP may be an example. We have obtained



TEXT-FIGURE 5.—Effect of duration of exposure to 2-OP, 10^{-3} M, on incorporation of ^{3}H-thymidine, ^{14}C-phenylalanine, and ^{3}H-uridine by human leukocytes—acute myelocytic leukemia (AML) *(left)* and 2 populations of phytohemagglutinin (PHA)-stimulated lymphocytes.

TABLE 1.—$^{14}CO_2$ production by human leukocytes

Human leukocyte population	Effect of 2-OP (% control)			
	^{14}C-1-glucose	^{14}C-6-glucose	^{14}C-UL-glucose	^{14}C-pyruvate
Mixed-normal	224	210	211	—
Mixed-normal	221	233	—	—
CLL*	600	—	310	
AML†	368	—	300	—
AML	—	—	—	104

*CLL = chronic lymphocytic leukemia.
†AML = acute myelocytic leukemia.

preliminary evidence that other differences exist as well. For example, at each 2-OP concentration and time studied with murine leukemic cells, protein synthesis was more inhibited than DNA synthesis; the reverse occurred when human leukemic cells were studied.

SUMMARY AND CONCLUSIONS

As a prelude to trials of propanals in human hematologic malignancies, we have examined the effects of 2-OP and DHP on human leukocytes *in vitro.* Effects on carbohydrate metabolism and on nucleic acid and protein synthesis were observed.

The observations with ^{14}C-labeled carbohydrates suggest that, even though sulfhydryl enzymes such as succinic dehydrogenase or glyceraldehyde phosphate dehydrogenase can be shown to be easily inhibited *in vitro* by osones such as 2-OP, they may not be the most sensitive sites of inhibition in intact cells. For example, oxidation of ^{14}C-6-glucose to $^{14}CO_2$ would be blocked by inhibition of any of several sulfhydryl enzymes required for the conversion; since this oxidation was not inhibited at 2-OP concentrations that inhibited RNA biosynthesis, the inhibition of sulfhydryl enzymes is unlikely to be the primary mode of action of 2-OP in the intact cell. This possibility stands in contrast to the results observed with DHP in the experimental animal *(11, 12)*. An intriguing observation is that 2-OP suppresses RNA biosynthesis in blastogenically transformed lymphocytes but not resting cells. This raises the question of whether certain aldoketones may function as immunosuppressive agents.

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Studies on the Effect of Antifolics in Mouse Leukemia[1, 2]

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Ladies and Gentlemen:

I am greatly honored to be here to present the results I achieved while participating in a group investigating the biochemical and biological action of antifolics and other drugs. This group and I observed and studied changes in the natural sensitivity and resistance of two strains of experimental leukemias during and after chemotherapy.

In brief, our method of estimation of the therapeutic effectiveness differs somewhat from the models described in the "Protocols for Screening Chemical Agents and Natural Products Against Animal Tumors and Other Biological Systems" edited by the Cancer Chemotherapy National Service Center. As a most important parameter indicating the effectiveness of drugs, we consider the prolongation of the average survival time of mice with leukemia. The principal schedule of the chemotherapy should consist of daily subcutaneous administration of the drugs. Text-figure 1 shows an idealized experiment indicating the results achieved with an effective drug. In the upper part is shown the prolongation of the survival, dependent on the size of the applied dose. Optimum doses cured the mice (arrows). In the lower part are shown changes in the weight of the mice and the changes in the weight of their spleens as compared to spleens of leukemic controls. The average weight of the dying mice treated with low doses of drugs did not differ from the initial weight. Administering higher doses normally caused an increase in the weight, because we usually worked with young, growing animals. After administering high toxic doses, we found a decrease in the average weight of the group. We also found similar changes in the weight of spleens of leukemic animals. In the mice surviving long-term therapy, we regularly found a greater increase in the weight of the spleens compared to that of leukemic controls, whereas, in mice given toxic doses, we found a lowering of the weight of the spleens.

If the drug is active, then a second characteristic to be determined is the therapeutic width, usually called the therapeutic index. This is a calculated ratio from the highest, maximally effective, and nontoxic dose to the lowest, daily administered dose which is the first dose to significantly prolong survival. I must add a note describing the exactness of the determination. In text-figure 1 are given two possibilities of choosing the first dose (A—full lines and solid circles; B—dashed lines and open circles). For simplicity, the first dose is always given as 1. We may read from the graph: Choice A gives the therapeutic index as 7.5, and choice B gives the therapeutic index as 5. Therefore, even though one is most precise in his work, one must remember the risk of a 50% error in such a case when the doses are increasing by the ratio of 1 to 1.5.

For example, I chose the results of therapy with 3′,5′-dibromoaminopterin (BrAP). Text-figure 2 shows the effect of this antimetabolite on L 14 AKR leukemia. The average survival of the animals increased with increasing daily dose. The longest survival occurred in mice given a daily dose of 17 mg/kg BrAP. When a higher dose was administered, survival decreased, the weight of spleens was lower than that of leukemic controls, and the weight of the group decreased, which all indicate toxicity *(1, 2)*.

The same therapy was tested, in collaboration with Dr. Souček, on the LaH VÚFB strain of mouse leukemia. Values in text-figure 3 are given in percents, as related to leukemic controls. As shown in text-figure 3, the toxic doses caused the same effect on LaH VÚFB as they did on Leukemia L 14 AKR, shown in text-figure 2. But we considered the prolongation of the survival too short to detect an increase in the weight of the spleens *(3)*.

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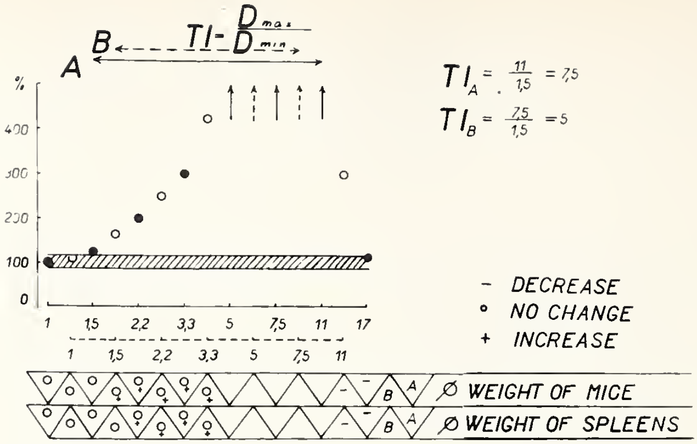
[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

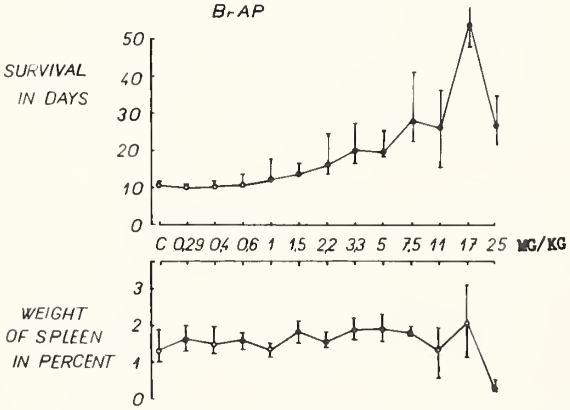
[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

[3] I thank Dr. K. Slavík, Dr. J. Souček, Dr. V. Slavíková, and Dr. K. Přistoupilová for their excellent collaboration.



TEXT-FIGURE 1.—An idealized result of a chemotherapeutic experiment.

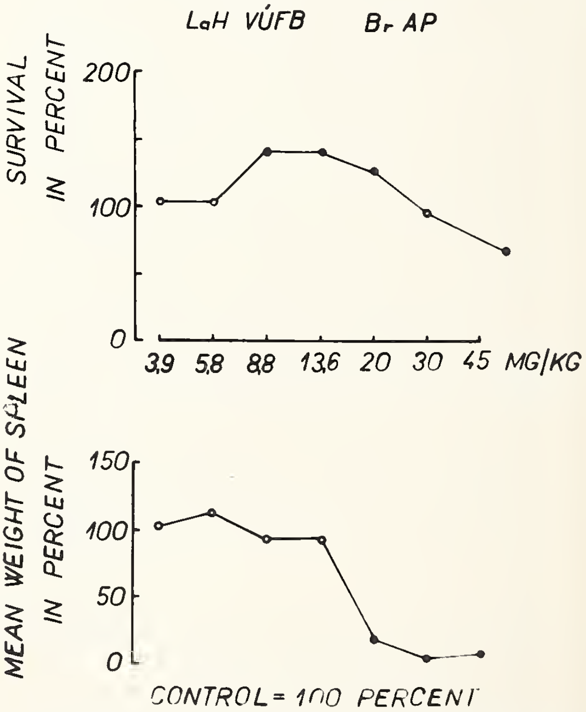
Using the above method, we propose Leukemia L 14 AKR to be an aminopterin (AP)-sensitive model and Leukemia LaH VÚFB to be an AP-resistant model. The results of the determination of the therapeutic effect of the drugs used for further experiments are given in table 1. Methotrexate (MTX) and BrAP prolonged the average survival of L 14 AKR leukemic mice 470 and 570%, respectively. Actinomycin D and vincristine cured L 14 AKR leukemia; *i.e.*, the affected mice treated for 50 days with optimal doses had not died 40 days after treatment was ended. However, Leukemia LaH VÚFB was much more resistant to therapy with AP, MTX, and BrAP. Survival from this leukemia was prolonged only by 7% (AP) up to 40% (BrAP). Actinomycin D prolonged survival by about 50%.



TEXT-FIGURE 2.—Result of chemotherapy of L 14 AKR leukemia with 3′,5′-dibromoaminopterin (BrAP).

It was of particular interest to us to ascertain the reasons for such differences in therapeutic responses between both leukemias. At that time, it was well known that the target enzyme for the 4-amino analogues of folic acid was folate reductase (dihydrofolate reductase).

Dr. Souček studied that problem in detail. In numerous experiments, he found many values which



TEXT-FIGURE 3.—Result of chemotherapy of LaH VÚFB leukemia with 3′,5′-dibromoaminopterin (BrAP).

TABLE 1.—Maximum average survivals from leukemias, with percentages of survival. Control = 100%

Survival achieved with	Leukemia			
	L 14 AKR		LaH VÚFB	
AP*	203	S†	107	N‡
MTX§	470	S	120	S
BrAP‖	570	S	140	S
Actinomycin D	Cure (in 80%)		150	S
Oncovin¶	Cure (in 60%)			

* Aminopterin.
† S = significant.
‡ N = not significant.
§ Methotrexate.
‖ 3',5'-Dibromoaminopterin.
¶ Vincristine sulfate, Lilly.

we correlated to the findings *in vivo*. But instead of finding an answer to our question, we found further differences. We followed up the inhibition of the liver enzyme *in vivo* with the toxicity of the 4-amino analogues of folic acid *(4)*. Table 2 shows the doses causing 50% inhibition of liver enzyme, estimated *in vitro* and *in vivo,* the doses causing the death of 50% of the mice, and the optimum therapeutic dose. The first line shows the micrograms of corresponding antimetabolite causing 50% inhibition of folate reductase *in vitro*. The second line gives the amount of antimetabolites required for the same degree of inhibition when applied to mice. Values for both AP and BrAP were relatively close. But the doses causing death of 50% of animals or those used in therapeutic experiments differed greatly between AP and BrAP. We concluded that the inhibition of folate reductase in liver could not be correlated with the therapeutic or toxic effects of AP or BrAP.

TABLE 2.—Inhibition of folate reductase *in vitro* and *in vivo*, LD 50%, and optimum therapeutic daily doses

	AP*	BrAP†
Inhibition *in vitro* (mg/liter)	0.0127	0.0147
In vivo (mg/kg)	0.0296	0.0234
LD 50% (mg/kg)	0.37	150
Optimum (mg/kg) therapeutic daily doses	0.11	17

* Aminopterin.
† 3',5'-Dibromoaminopterin.

Now I shall deal with toxicity. There were many discussions about the differences in data on toxicity given in the literature. Table 3 has been taken from one of our publications which arose from such a discussion *(5)*. We used 3 groups of mice of the same origin but of different ages and weights. The toxicity of AP depended on the age of the mice. The heaviest mice were adult, 6–7 weeks old; the others were 4 and 3 weeks old, respectively. The toxicity of AP for adult mice was 6 times less than that for growing mice. From this and other experiments, we deduced that the exactness of the determination of the toxicity depends on many factors and that, for the therapeutic width (or index), it is better to correlate the results achieved in one group of animals.

TABLE 3.—Dependence of toxicity of aminopterin on the age of H ♂ mice

Initial weight of mice (g)	Dose (mg/kg)	Dead/total
12–16	0.5	9/10
	1.0	10/10
	1.5	10/10
	2.0	10/10
	2.5	10/10
16–20	0.5	8/10
	1.0	10/10
	1.5	10/10
	2.0	10/10
	2.5	10/10
21 or more	0.5	0/10
	1.0	2/10
	1.5	2/10
	2.0	5/10
	2.5	5/10

But now I shall return to the subject of the inhibition of folate reductase. The inhibition of this enzyme exerted by AP decreased during 1 week from 90% to about 50%. At that value, the activity of folate reductase persisted minimally for another week. An example demonstrating this fact is shown in table 4 (col. A). If we injected AP or BrAP at low doses into mice, then 50% inhibition persisted for over 14 days. But, if we injected AP and BrAP at high doses, then the inhibition caused by AP did not decrease during 3 consecutive days. No animal in the experiment survived up to the time the assay was performed after 14 days. If we injected the same amount of BrAP, then the inhibition decreased over 88% on the 3d day to 63% on the 14th day. This was the first difference found between AP and BrAP.

There was also another group of animals shown in table 4; this group was not treated with antimetabolites but was kept in the same cages with the treated mice (col. B). In these animals, we also found inhibition of folate reductase, although we placed food in a separate experiment so that it could not be contaminated with fecal matter (asterisk in table 4). In experiments with mice, one has to take into account the mouse's coprophagy and self-washing when one is considering the persistence of the inhibition of an enzyme caused with a chemically stable drug.

TABLE 4.—Inhibition of folate reductase by aminopterin (AP) and 3',5'-dibromoaminopterin (BrAP)

Daily dose (mg/kg)	Antimetabolite	Inhibition of folate reductase in percent after					
		24 hours		3 or 4 days		14 days	
		A	B	A	B	A	B
0.15	AP	86	0	92	7	50	0
0.15	BrAP	82	7	95	26	51	30
15	AP	96	91	100	93	Dead	55
15	BrAP	90	60	88	62	63	56
15	BrAP*	88	88	88	86	—	—

* One experiment in which we placed food so that it could not be contaminated with fecal matter.

TABLE 5.—Activity of folate reductase

Mouse strain	Antimetabolite	Dose (mg/kg)	Percent of activity			
			Embryo		Liver	
			2 hours	24 hours	2 hours	24 hours
H	AP*	0.25	61	69	0	0
C57BL			100	60	0	0
H	BrAP†	0.25	100	100	0	0
C57BL			100	100	0	0
H	AP	22.5	0	0	0	0
C57BL			0	0	0	0
H	BrAP	22.5	0	22	0	0
C57BL			0	50	0	0

*AP = aminopterin.
†BrAP = 3',5'-dibromoaminopterin.

Initial results correlating the activity of the folate reductase with the toxicity were found in the inhibition of folate-reducing enzyme in mouse embryos *(6)*. Table 5 shows that low doses of both AP and BrAP inhibited the activity of liver enzyme in about the same way. Although no difference was found in behavior of the liver and embryonic enzymes *in vitro*, two differences were found when antimetabolites were injected into the animals. The first difference was that low doses of AP, but not those of BrAP, partially inhibited folate reductase in embryos. The other difference was that embryonic folate reductase inhibition decreased 24 hours after administration of BrAP but not after AP. Clearly, the activity of the target enzyme, folate reductase, was not equally inhibited in liver and embryo taken from an animal even after administration of one antimetabolite.

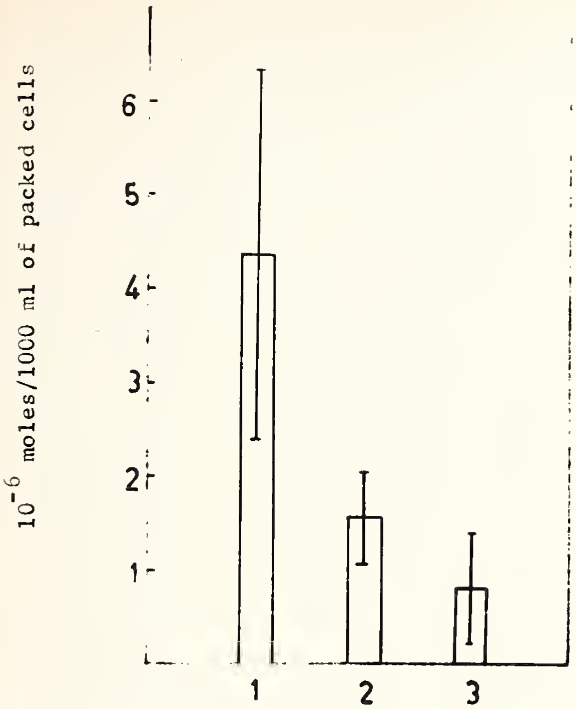
Further experiments were done with Ehrlich ascites tumor cells (table 6). Dr. Přistoupilová incubated these cells in the same molar concentrations of AP, MTX, and BrAP *(7)*. In the same volume of analyzed cells, the most toxic of these drugs, AP, accumulated in the largest amount, followed by MTX and then BrAP which was found in minimal amounts at the time that the reaction was stopped.

TABLE 6.—Concentration of antifolics in Ehrlich ascites tumor cells *in vitro*

Incubation at 37°C	Antimetabolite	10^{-6} moles in 1000 ml of packed cells
10 minutes	AP*	1.47, 1.41
	MTX†	0.52, 0.7
	BrAP‡	0.29, 0.23
40 minutes	AP	2.54, 2.57
	MTX	1.3, 1.47
	BrAP	0.41, 0.45

* AP = aminopterin.
† MTX = methotrexate.
‡ BrAP = 3',5'-dibromoaminopterin.

The determination of the amount of antifolics in Ehrlich ascites tumor cells 2 hours after administration of 2.56 × 10^{-7} moles of AP, MTX, and BrAP into mice revealed similar relations (text-fig. 4). The largest amount of an antimetabolite was found in the cells from animals treated with AP, much less in cells from animals treated with MTX, and the least in cells from animals treated with BrAP. Further results showed that the efflux studied *in vitro* differs among these 3 antimetabolites. AP persisted in Ehrlich ascites cells for the longest time; the efflux of MTX and BrAP was faster.



Text-figure 4.—Amount of antifolics in Ehrlich ascites tumor cells 2 hours after administration of 2.56 × 10^{-7} moles of aminopterin (col. 1), methotrexate (col. 2), and 3′,5′-dibromoaminopterin (col. 3) into mice.

From these results, we conclude: Although AP, MTX, and BrAP inhibited the enzyme folate reductase on the same molar basis, biological factors regulating transport, accumulation, and perhaps decomposition of the drugs or drug-enzyme complexes caused differences in biological effects. When we consider the inhibition of folate reductase as causing toxicity, then, according to the duration of their persistence in the cells, AP exhibits the inhibition for the longest period and BrAP for the shortest period.

When we studied the folate-reducing enzyme, we proved also the activities of enzymes which transform tetrahydrofolic acid into its coenzyme forms and we investigated the biosynthesis of purines and methionine in liver of normal and leukemic mice. In addition, we studied the influence of BrAP and actinomycin D therapy on the activities of these biosynthetic pathways *(8)*. Table 7 shows that the activities of tetrahydrofolic acid formylase and formiminotransferase in liver of leukemic animals on the 7th day of the disease were lower than those found in normal animals; this comparison was based on the wet weight of the organs. Administering therapeutic doses of BrAP and actinomycin D did not substantially change the activities of those enzymes in normal mouse liver. The decrease in activities of the formylase and formiminotransferase was slight or not present in treated leukemic L 14 AKR mice. In LaH VÚFB leukemic mice, the decrease in activities of these enzymes was less inhibited than that in L 14 AKR leukemic mice, which corresponds with observed weaker response to therapy with both drugs in mice with LaH VÚFB leukemia. The last enzyme shown in table 7, N^{5-10}-methylenetetrahydrofolic acid dehydrogenase, underwent no significant changes, as calculated on the basis of wet weight of liver.

The activities of these enzymes in the spleens of nonleukemic mice were very low compared to those in the liver of normal mice. In the spleens of leukemic animals, the activities of formylase and dehydrogenase, but not formiminotransferase, were

Table 7.—Activities of the enzymes of tetrahydrofolic acid formylase, formiminotransferase, and N^{5-10}-methylenetetrahydrofolic acid dehydrogenase in the livers of normal and leukemic mice (L 14 AKR, LaH VÚFB)

	Formylase		Formiminotransferase		Dehydrogenase	
Normal mice	AKR	C57BL	AKR	C57BL	AKR	C57BL
Control group	100 (2.86 ± 0.7)	100 (1.92 ± 0.2)	100 (5.0)	100 (3.14 ± 0.25)	100 (3.14 ± 0.21)	100 (4.0 ± 0.06)
BrAP*	96.2 ± 8.0	98.5 ± 6.0	95.3	99.8 ± 4.0	95.5	98.0 ± 2.0
Act. D†	88.0 ± 10	92.0 ± 11	104	99.0 ± 8.0	96.5	99.5 ± 1.0
Leukemic mice	L 14 AKR	LaH VÚFB	L 14 AKR	LaH VÚFB	L 14 AKR	LaH VÚFB
Control group	62.8 ± 16	63.5 ± 12	73.6	78.5 ± 13	95.0	99.5 ± 1
BrAP	91.5 ± 16	60.7 ± 20	92.0	83.0 ± 10	97.0	98.0 ± 2
Act. D	100.0 ± 1	84.0 ± 9	104.0	98.4 ± 5	97.7	99.5 ± 1

* BrAP = 3′,5′-dibromoaminopterin.
† Act. D = actinomycin D.

higher than those in nonleukemic animals; however, they did not achieve the activity of a normal mouse liver. Therefore, when we considered the differences in the weight, cellularity, and enzyme activities, as well as the effect of chemotherapy, we concluded that the apparently decreased activity of enzymes in livers of leukemic animals may have been caused by infiltrating leukemic cells.

The rate of biosynthesis of methionine and inosinic acid was elevated in livers of leukemic mice. Administering actinomycin D decreased the rate of biosynthesis of inosinic acid in livers of normal mice. As table 8 shows, BrAP and actinomycin D retarded the rate of enzymatic syntheses in Leukemia L 14 AKR. They affected the enzymatic activities in LaH VÚFB leukemia to a much lesser extent, however.

TABLE 8.—Incorporation of ^{14}C-3-serine into methionine and inosinic acid in the livers of normal and leukemic mice (L 14 AKR, LaH VÚFB)

	Methionine		Inosinic acid	
Normal mice	AKR	C57BL	AKR	C57BL
Control group	100 (933 ± 323)	100 (1535 ± 564)	100 (1179 ± 177)	100 (1111 ± 156)
BrAP*	101 ± 14	96 ± 31	94	110
Act. D†	103 ± 3	100 ± 14	79 ± 3	55 ± 19
Leukemic mice	L 14 AKR	LaH VÚFB	L 14 AKR	LaH VÚFB
Control group	268 ± 86	183 ± 65	155 ± 6	216 ± 65
BrAP	150 ± 27	143 ± 40	116	122
Act. D	91 ± 40	142 ± 14	89	181 ± 29

* BrAP = 3',5'-dibromoaminopterin.
† Act. D = actinomycin D.

During therapeutic experiments, we also investigated how long the effective concentrations of administered drugs persist in mice *(9)*. To determine the persistence of the therapeutic effective level, we administered an inoculum of experimental leukemia at certain intervals after effective drugs had been given. One result is shown in table 9. Ninety minutes after treating the animals with BrAP or MTX at a daily dose slightly higher than optimal, we found a significant prolongation of the lifespan of mice with L 14 AKR leukemia. Smaller doses of both drugs did not prolong the survival of the animals, as evaluated statistically. But, to our surprise, actinomycin D did not significantly prolong survival, even if it was administered simultaneously with the leukemic L 14 AKR cells, though some animals lived for a long time. So the most effective drug, the drug which cured L 14 AKR leukemic mice *(10)*, did not exert an effect like that of the 4-amino analogues of folic acid, which only prolonged the life of the leukemic animals.

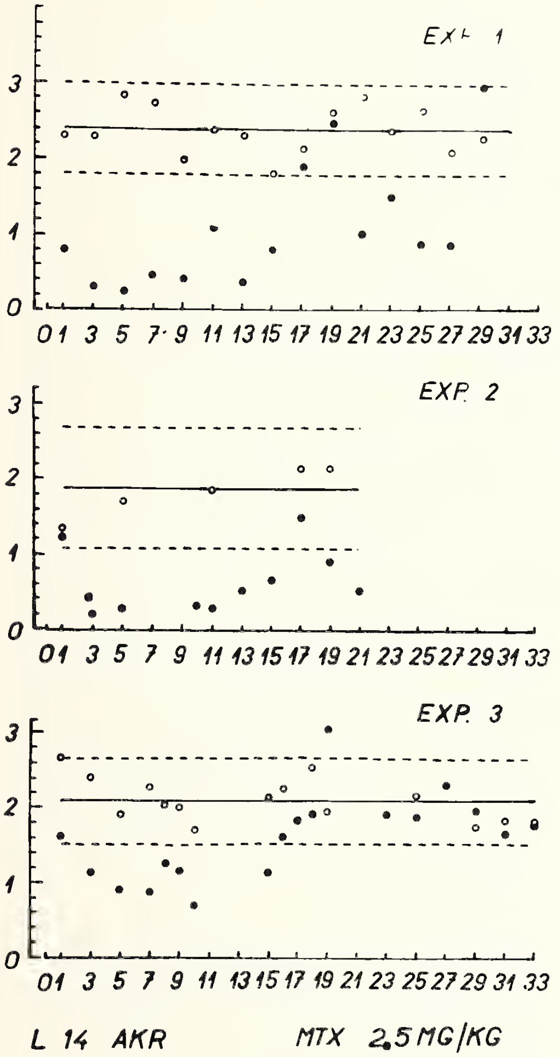
For further experiment, we formulated the following question: Could we see exactly on growing neoplastic cells what happens after one dose of an effective drug is administered? To answer this question, we searched for changes in imprints of leukemic liver *(11)*. We followed the frequency of mitoses in animals killed at certain intervals after the drug had been given. Text-figure 5 shows the results achieved in mice with L 14 AKR leukemia

TABLE 9.—Prolongation of the survival of AKR mice* treated with 3',5'-dibromoaminopterin (BrAP), methotrexate (MTX), or actinomycin D (Act. D) 90 minutes before inoculation of L 14 AKR leukemic cells

Dose (mg/kg)	Survival (variation)	Significance
Control group	8.26 (8–9)	
BrAP:		
6.65	8.62 (8–10)	N†
20.0	9.38 (8–11)	0.01
MTX:		
0.66	8.83 (8–14)	N
2.0	9 (8–10)	0.01
Act. D:		
0.033	8.46 (8–11)	N
0.1	10.1 (8–25)	N

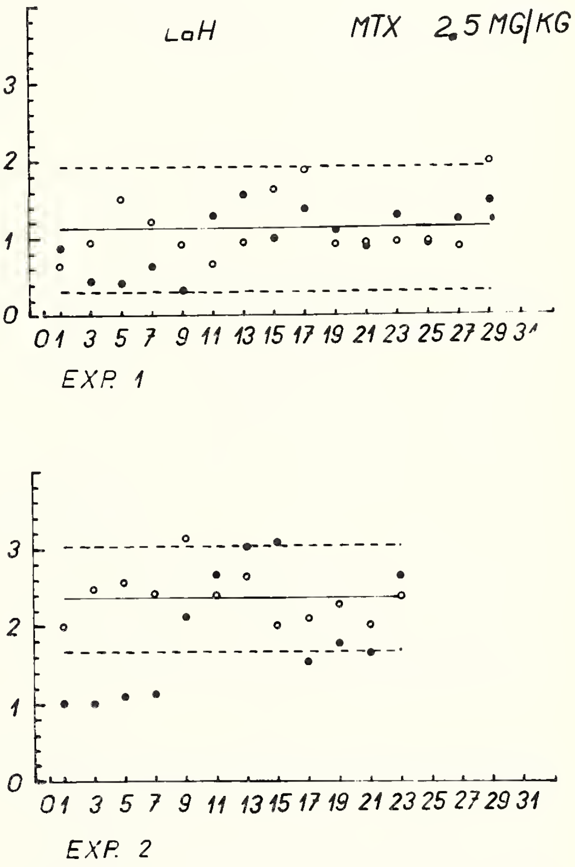
* In each group were 18 or 19 mice.
† N = not significant.

which were given 2.5 mg/kg MTX on the 5th day of the disease. The mitotic indexes decreased, beginning between the 1st and the 3d hour and lasting until the 15th hour after drug administration. Regularly following this was a second drop, but not as steep as the first one. In that L 14 AKR leukemia sensitive to AP, we observed in liver imprints of animals (which were usually killed 7, 9, and 11 hours after drug administration) leukemic blasts damaged to different degrees. Possibly some of these cells had been destroyed by the drug.



Text-figure 5.—Changes in the frequency of mitoses after one dose of methotrexate (2.5 mg/kg). *Solid circles*—treated L 14 AKR leukemic mice. *Open circles*—control L 14 AKR leukemic mice. The arithmetical mean of control values *(solid line)* is demarcated by *broken lines* indicating 2 SD.

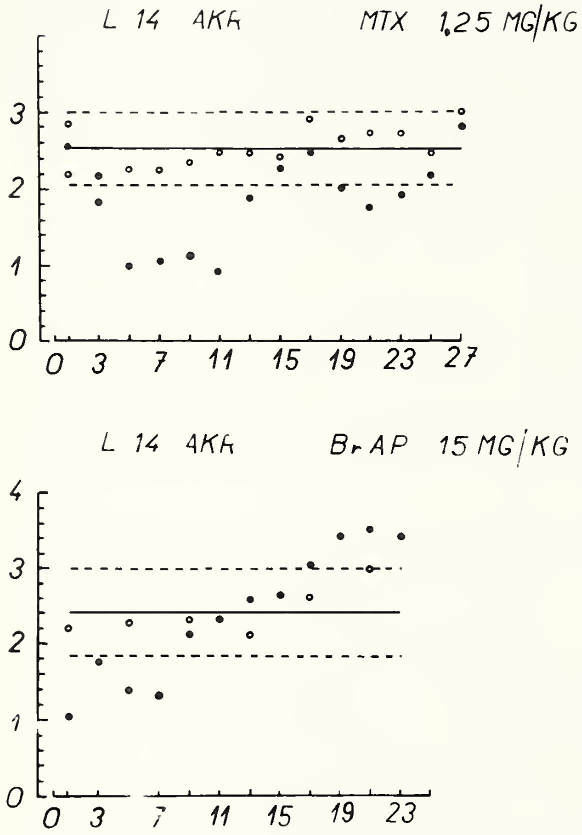
In text-figure 6, the effect of the same dose of MTX, 2.5 mg/kg, is described. We administered MTX to leukemic C57BL mice. LaH VÚFB leukemia, which is carried in the C57BL strain, is naturally AP resistant. The therapy with MTX increased the survival by only 20%. Considering the decrease in mitotic frequency as indicating the rate of cell reproduction, we saw that only 4 values, representing 7 hours after administration of MTX, demonstrated a decrease in mitosis. Nevertheless, the drop was not so steep as that observed in the L 14 AKR leukemic mice.



Text-figure 6.—Changes in the frequency of mitoses after one dose of methotrexate (2.5 mg/kg). *Solid circles*—treated LaH VÚFB leukemic C57BL mice. *Open circles*—control LaH VÚFB leukemic mice.

Text-figure 7 shows the effect produced when the dose of MTX used was halved to 1.25 mg/kg and administered to mice with L 14 AKR leukemia. The mitotic indexes decreased from the 3d to the

13th hour after treatment; this drop was approximately twice as long and steep as that seen in the AP-resistant LaH VÚFB leukemic mice given 2.5 mg/kg MTX. Text-figure 7 also indicates that a second smaller drop occurred, lasting from the 19th to the 23d hour after treatment.



Text-figure 7.—Changes in the frequency of mitoses after one dose of methotrexate (MTX) (1.25 mg/kg) and 3′,5′-dibromoaminopterin (BrAP) (15 mg/kg).

In the lower part of text-figure 7 is shown the effect of 15 mg/kg BrAP. The time course of the depression of mitotic indexes induced by BrAP was not the same as that seen after MTX treatment. With BrAP, the mitotic index was lowered for only 7 hours but then returned to normal. Seventeen hours after application of BrAP, the frequencies of mitoses rose, which lasted to the end of the experiment.

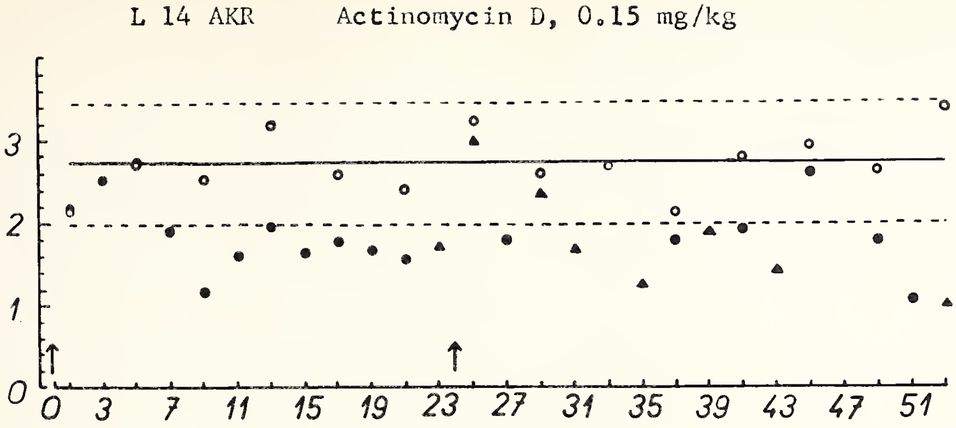
A very different result was found after application of actinomycin D (text-fig. 8). The first value of mitotic index decreasing below the border of significance was observed in the 7th hour after the drug administration. Then the mitotic indexes remained below the border of significance during the 1st day of the experiment. On the 2d day, although the drug was again applied (as indicated by the arrow in text-fig. 8), a more distinct drop in mitotic indexes did not occur. But the number of leukemic cells decreased (as indicated in text-fig. 8 by triangles).

The decrease in mitotic frequencies observed after administration of drugs we consider a sign of a decreased rate in cell reproduction. With MTX, we presumed that this decrease was caused by two factors: 1) killing of a part of the malignant cell population, and 2) a transient prolongation of the interval between two successive cell divisions.

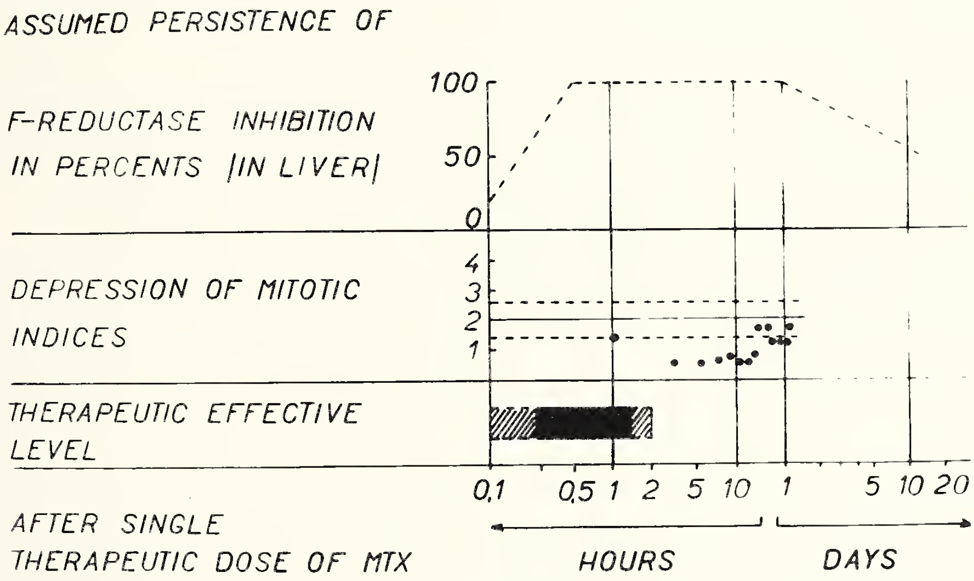
We did not prove all three phenomena which we supposed were the most important effects of MTX administration in one experiment; therefore, the data in text-figure 9 were deduced from the results of separate experiments. At the top of text-figure 9, the time course of folate-reductase inhibition is shown as observed in normal mouse liver. The middle part of the text-figure indicates the changes in mitotic indexes of leukemic blasts after a single dose of MTX. The normal distribution of frequencies of mitoses in unaffected leukemic animals is indicated by the strip along the mean value. The bottom part of the text-figure represents the persistence of the effective level after administration of one dose of MTX. Time is indicated here in logarithmic scale—to the left in hours and to the right in days. We supposed that the inhibition of folate reductase and the appearance of a therapeutic level started at the same time. We could observe the consequences of these factors, *i.e.,* decreased rate of reproduction of cells as indicated by lowered amount of mitoses, when the effective level was disappearing. The mitotic frequency returned to normal when a small folate-reductase activity could be seen.

There was another question: Could we prove a potentiation in therapeutic responses by exploring the above-mentioned statement and correlating the time of drug applications with the changes in mitotic activities of leukemic cells?

A single dose of MTX (text-fig. 10), if administered on the 1st through the 7th day of the disease, caused statistically significant prolongation of survival in the treated groups compared to the control group; the treated groups were indisting-



TEXT-FIGURE 8.—Changes in mitoses after repeated administration of actinomycin D (0.15 mg/kg). *Open circles*—leukemic controls. *Solid circles*—treated leukemic mice. *Arrows*—time of administration of the antibiotic. *Triangles*—for the depicted values, the required number of leukemic cells could not be found in imprint.

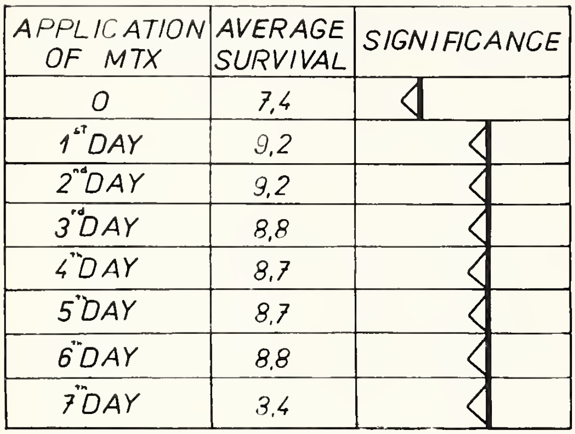


TEXT-FIGURE 9.—Assumed time course of liver folate-reductase inhibition, depression of mitotic frequencies, and persistence of therapeutic effective level after one dose of methotrexate (MTX).

uishable from each other. In the following experiment, we treated the L 14 AKR leukemic mice with 2 doses of MTX. As text-figure 11 indicates, 2.5 mg/kg MTX, administered once or twice simultaneously (during a 5-minute interval), significantly prolonged the survival in the treated groups compared to that of the control group. But, if we delayed the administration of the second dose by 9 or more hours, then the survival was significantly prolonged in comparison to the doubled simultaneously administered dose. In further experiments, we studied the prolongation of the survival by applying the second dose of MTX at the time when we expected that the mitotic activity had returned to normal values (17 hours after the first dose) or at a later time when the second drop in mitotic frequency was observed (20 or 23 hours after the first dose). As indicated in text-figure 12, we found no significant differences if the injection of MTX was repeated after 17, 20, or 23 hours.

In further work we suppose that, coincidental with the observed decreases in mitotic activity, we will find the changes in utilization of deoxyribonucleic or ribonucleic acid precursors. Hopefully, if

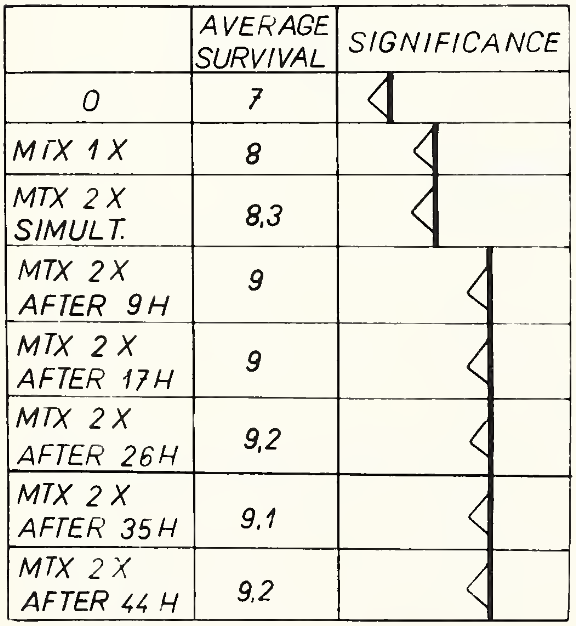
SURVIVAL AFTER ONE DOSE OF MTX
(2.5 mg/kg)



APPLICATION OF MTX	AVERAGE SURVIVAL	SIGNIFICANCE
0	7,4	◁
1st DAY	9,2	◁
2nd DAY	9,2	◁
3rd DAY	8,8	◁
4th DAY	8,7	◁
5th DAY	8,7	◁
6th DAY	8,8	◁
7th DAY	8,4	◁

Text-figure 10.—Prolongation of the survival of leukemic L 14 AKR mice after 1 dose of 2.5 mg/kg methotrexate (MTX).

REPEATED APPLICATION OF MTX
(2.5 mg/kg)

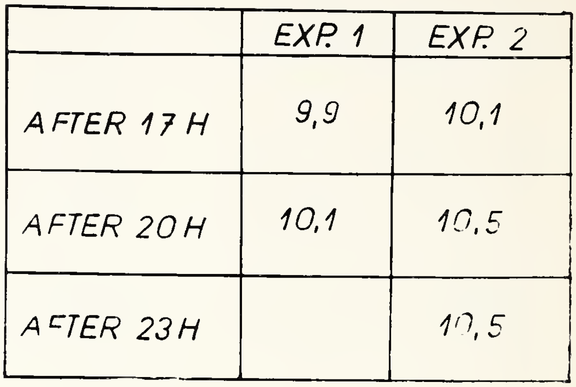


	AVERAGE SURVIVAL	SIGNIFICANCE
0	7	◁
MTX 1 X	8	◁
MTX 2 X SIMULT.	8,3	◁
MTX 2 X AFTER 9 H	9	◁
MTX 2 X AFTER 17 H	9	◁
MTX 2 X AFTER 26 H	9,2	◁
MTX 2 X AFTER 35 H	9,1	◁
MTX 2 X AFTER 44 H	9,2	◁

Text-figure 11.—Prolongation of the survival of leukemic L 14 AKR mice after 1 or 2 doses of 2.5 mg/kg methotrexate (MTX).

we understand better these observed changes in mitotic frequency, we can achieve a further distinct potentiation of the therapeutic effect of cytotoxic drugs.

REPEATED APPLICATION OF MTX (2.5 mg/kg)



	EXP. 1	EXP. 2
AFTER 17 H	9,9	10,1
AFTER 20 H	10,1	10,5
AFTER 23 H		10,5

DIFFERENCES NOT SIGNIFICANT

Text-figure 12.—Difference in the survival of leukemic L 14 AKR mice after repeated administration of 2.5 mg/kg of methotrexate (MTX).

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Prediction of Methotrexate Responsiveness of Tumors in Man[1, 2]

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NEOPLASMS of man may be naturally resistant to folate antagonists or acquire resistance after a period of sensitivity. Studies have been performed on patients with acute myelocytic leukemia (AML), as an example of a malignancy not usually responsive to conventional therapy with methotrexate (MTX), and on patients with acute lymphoblastic leukemia (ALL), as an example of a malignancy usually initially responsive which then develops resistance to MTX *(1)*. Since MTX blocks DNA synthesis by inhibiting dihydrofolate reductase, an enzyme necessary to generate methylene tetrahydrofolate for thymidylate biosynthesis, this enzyme activity, as well as the incorporation of ^{3}H-deoxyuridine (UDR) and ^{3}H-thymidine (TDR) into DNA, was measured in patients before, during, and after therapy. No difference was detected in blast cells from patients with AML compared to those from patients with ALL in regard to the level of dihydrofolate reductase activity, inhibition of the enzyme by MTX, rate of incorporation of UDR or TDR into DNA, or inhibition of UDR incorporation into DNA by 10^{-5}–10^{-8} M MTX. However, when MTX was administered to patients with these types of leukemia and dihydrofolate reductase activity and DNA synthesis were monitored by measuring UDR and TDR incorporation into blast cell DNA, inhibition of dihydrofolate reductase and of UDR into DNA was greater in cells of patients with ALL than in those of patients with AML. These results correlated well with the outcome of therapy. They may be due to the longer generation time and the faster turnover rate of dihydrofolate reductase in AML cells than in ALL cells. This suggestion is supported by the rise in total enzyme (free plus MTX-bound) noted in extracts of cells from patients with AML as compared to those with ALL 24 hours after MTX treatment.

Patients with ALL who acquire resistance to MTX have been found to have either "schedule" resistance, which cannot be detected by an *in vitro* assay, or "biochemical" resistance, which can be detected by measurement of the effect of MTX on UDR incorporation into DNA. Conclusive evidence has not yet been obtained to indicate that this biochemical resistance occurs by selection of mutant cells with defective transport of MTX or with an increase in the level or an altered dihydrofolate reductase with less affinity for MTX. Acquired resistance in ALL may be due therefore to selection of mutant cells with *(a)* a more rapid turnover of enzyme, or *(b)* longer generation times, or *(c)* other mechanisms not yet elucidated.

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[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

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DISCUSSION

Chairman: Dr. Hall and Dr. Kessel, I was wondering about the qualities of your remission; the plus just is not enough for me. I wanted to know whether there was a day, 2 days, or something significant.

Dr. Hall: These qualities of remission were as defined by leukemia group B.

Dr. Fischer: Dr. Bertino, since methotrexate (MTX) currently protects the enzyme from degradation, might that not occur in the cell? Have you determined total enzyme and total dihydrofolic reductase activity, which would be very important to know?

Dr. Bertino: We have not, but this would be worthwhile doing. I tried to derive some way of ascertaining total enzyme from the *p*H 8.5 versus *p*H 7 activity, but it got very complicated and was accurate only over a small range. We really could not do it. We made an approximation, which included most of the enzyme but not a complete total.

Dr. Heidelberger: The work with dihydroxypropanal is somewhat reminiscent of the considerable clinical work done in Italy with dihydroxybutyraldehyde in which it seems quite clear that the main mode of action was inhibition of protein synthesis as the result of sulfhydryl inhibition. There is quite a striking similarity there.

I would like to congratulate Dr. Hall and Dr. Reyes who discovered a 5-fluorouracil (5-FU) pathway that we missed. I was also pleased to see that they confirmed work we published 10 years ago showing a concentrated uptake of 5-FU in the cells; we felt that this was a pump rather than a diffusion type of thing. In the patients, you showed an apparently high level of phosphorylation of fluorouridine (FUR) as compared with 5-FU. It is possible that the phosphorylation may just reflect the increased uptake of the drugs into the cells? We showed some time ago that 5-FU was converted mostly into ribonucleotides and then also into the deoxyribonucleotide monophosphate, 5-fluoro-2′-deoxyuridine phosphate (FUdRP), the active drug. Dr. Kessel and Dr. Hall showed very beautifully and convincingly in a series of mouse leukemias that the activity of the compound was related to the total conversion to nucleotides. At a meeting held in Washington about 1 month ago on 5-FU, Dr. Rogers indicated that, in the early periods after administration of the drug, the nucleotides in the tumors were primarily the ribonucleotides. But, after long periods of up to 48 hours, the only nucleotide remaining in the cell was FUdRP, the active form of the drug. Apparently 5-FU has not been adequately tested in leukemias. Although most clinicians have scoffed at this, I finally received support at this meeting. We need a clinical trial of 5-FU in acute leukemias.

Finally, this inverse relationship between thymidine kinase and the response of these mouse leukemias is not quite as paradoxical as it might seem at first sight. The reason for this is the great efficiency with which FUdRP inhibits thymidylate synthetase. Half maximum levels, at least with one mouse enzyme, are at about 10^{-7} M. Probably this means that the small amount that does get phosphorylated by thymidine kinase is sufficient to produce an impressive blockade of thymidylate synthetase because the ratio is somewhat over 1,000. It means essentially that 1 molecule of FUdRP will block the passage of 1,000 molecules of deoxyuridylate.

Dr. Hall: The data on FUR are consistent with the concept that there is a transport pump which is poorly saturated at therapeutic drug concentrations; for 5-FU, passive diffusion seems to operate. The problem is that phosphorylation occurs so rapidly that we cannot separate intracellular accumulation of nucleotides and retention from active transport. What we need is an FUR-resistant mutant based on a phosphorylation defect which we do not have.

Dr. Kessel: We found the same thing in uridine as well as in FUR: During a very short-term incubation, in the uptake with 5-FU, fluoroorotic acid, 5-fluro-2′-deoxyuridine (FUDR), and the corresponding nonfluorinated compounds, the concentration inside the cell and outside the cell is the same. But with uridine and FUR, the concentration inside is about 50-fold higher than that in the medium.

Dr. Hall: You must have a mutant in order to sew it up. With cytosine arabinoside, you have to make a mutant deficient in deoxycytidine kinase, and unfortunately we do not have an FUR-resistant mutant. I hope that, when we do the 5-FU trial in leukemia in man and find that it does not work, we do not stop this work, because we will then find that it does work with FUR. The pathway between 5-FU and 5-fluorouridine monophosphate is not the critical problem in leukemia; it is the deletion of uridine phosphorylase. In all leukemia trials of which I am aware, FUDR has been given almost always by short pulses so that one can insure its conversion to 5-FU. However, there were 2 exceptions to this method of FUDR administration: intra-arterial infusion of FUDR and, in the series reported by Moertel and by Sullivan and Miller, prolonged, intravenous infusion of FUDR. It was repeatedly stated that, if one gives FUDR by prolonged, intravenous infusion, one decreases the amount required by 100-fold. The reason for this is that, if one gives FUDR by slow infusion, one allows it to be a substrate for thymidine kinase and one makes a larger fraction of FUdRP. If one gives a large dose, FUDR all goes back to 5-FU and there is no way in which it can be brought back to the nucleoside again.

Dr. Bertino, your conclusions seem to be inconsistent: First you said you could not make correlations; then you showed us many examples which substantiated the correlation you could not make.

Dr. Bertino: The correlation was not with the *in vitro* test before treatment. It was with the degree of inhibition achieved and the duration of the inhibition during treatment. In other words, not all patients who had 3 logs of inhibition in the *in vitro* test actually had that many *in vivo*.

Dr. Hall: In our experience with single doses, a good way to test for response is to have a single dose of 20 mg MTX and then, 24 hours later, test response. Those patients who had an excess of MTX in the cells, more than enough to inhibit reductase and more than enough to inhibit deoxyuridine incorporation into DNA, seemed to do better in that test. But the normal bone marrow and acute granulocytic leukemia patients did not have complete inhibition and suppression of the enzyme.

Dr. Fischer: The transport of MTX has not been studied adequately. The initial rate of transport, for example, would be a very important parameter. However, this is a two-way path. But, since the enzyme probably dissociates from the drug to some extent within the cell, possibly a transport mutant with a very effective output physiologically can remove MTX from the cell; this would be just a slight additional parameter in a transport study. In other words, how well do the leukemic patients retain MTX once it enters? It certainly is a very real parameter; *e.g.*, a transport mutant that had normal levels for inhibition of cell growth and culture could pump MTX away from the enzyme if its out-pump was quite effective. The affinity for the out-pump was higher than that for the enzyme. The leukemic cells could not retain the drug. This was seen in some granulocytes we examined.

Dr. Bertino: These data indicate that the way to treat acute myelocytic leukemia would be to have longer infusions; of course, when you have longer infusions, you start getting into trouble. You might be able to go 48 hours with a reasonably good dose and perhaps do better, and that is about the limit you can get. In terms of prolonged low levels, that would be a very effective way of treating acute lymphatic leukemia (ALL), because that cell type perhaps does accumulate MTX in excess, which is needed to inhibit the enzyme and DNA synthesis. In this way, you may be able to get remission without any toxicity.

You could inhibit the enzyme in ALL very easily with low doses and get prolonged enzyme inhibition. When we started this, our object was really to monitor the patient and to use only that drug and only that duration necessary to achieve remission. We thought about doing that with some patients—just continuing until that bend in the thymidine went down. This is a reasonable way to approach monitoring MTX therapy.

Dr. Hall: As Dr. Bertino said, resistance is to a particular dose schedule. Resistance to oral MTX for maintenance can be avoided by giving MTX parenterally; this is one of the great contributions from acute leukemia group B. It is not only a matter of the amount or the dose schedule, but it is also a matter of the point of the natural history of the patient. Most of your patients probably were previously untreated. When you said transport is not critical, this is probably so. But, if we look at patients who have relapsed on drug therapy, then transport will be found to be important. Untreated ALL's transport MTX well, but they surely have the capacity to delete this mechanism, and the clones that grow out under the pressure of the drug surely have poor transport.

PANEL VI

STUDIES WITH SOLID TUMORS

Studies With Analogues of Purine Nucleosides[1, 2]

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THE development of test systems for drug sensitivities in solid tumors can be expected to meet with more problems than are evident in the comparable studies of leukemias. The samples obtained from solid tumors will probably be more heterogeneous. The "growth fraction" of the tumor is likely to be relatively small, a factor that is important when most of our test drugs are active on the reactions prevalent in growing cells, the nucleic acid and protein synthesis. Whereas the sampling of leukemic marrows can rather readily be scheduled, solid tumor biopsies are usually available only at the time of surgery, the scheduling of which may not lend itself to immediate testing of biopsy specimens in the laboratory. This circumstance should lead us to attempt to base our drug sensitivity tests on systems that can be studied in cell-free extracts of the tumors after freezing and storage of the samples. Design of the tests should perhaps tend toward somewhat indirect systems that intercept the line of synthesis involved in the effect of the drug at a point such that cells, not in DNA synthesis, will be involved in the measurements of a drug having its important effect on DNA synthesis.

It is quite evident that the more information we have concerning the metabolic effects of the drug, its activation, inactivation, excretion, and potential resistance mechanisms, the more feasible is the design of a test system able to predict favorable responses. At this relatively early stage, somewhat imperfect systems are likely to evolve. These will require refinement, but even imperfect systems would be an improvement over the present situation. As it is now, we only know that a certain relatively small percentage of mammary carcinomas can be expected to respond to drug X, for example. To measurably increase the percentage of responses in treated patients by selection of the cases would be progress.

Establishing the value of such predictive tests necessarily involves clinical trials of the drugs in parallel with the potentially predictive test systems. My available data have not yet included such parallel tests but have included what might be regarded as feasibility studies that serve to illustrate some principles.

One example we have studied is the purine nucleoside analogue, arabinosyl-6-mercaptopurine (Ara-6-MP). This drug was found to be carcinostatic in a spectrum of mouse neoplasms *(1–3)*. Relatively high and frequent dosage was required to inhibit bone marrow in the mouse. This drug seems to represent an unusually simple case for investigation of correlative tests. Although it is a purine analogue, its only observed metabolic effect at carcinostatic dosage was an inhibition of the cytidylate reductase of responsive mouse tumors. Unlike most purine analogues, it did not require activation by conversion to nucleotide and was not appreciably metabolized to free base *(1–4)*. It apparently was active as such, and on only one specific reaction. Urinary excretion by treated mice was moderately rapid, 75% in 6 hours, and was largely (88%) as unchanged Ara-6-MP.

The glutamine antagonist, azaserine, produced synergistic responses in combination with Ara-6-MP against the Ehrlich ascites carcinoma, an effect that seemed to be logically explained as a sequential inhibition, since azaserine inhibited the conversion of uridylate to cytidylate in these cells and decreased the cytidylate pools, whereas Ara-6-MP inhibited the cytidylate reductase *(2)*. The effect observed *in vivo* was a decrease in the labeling of DNA cytosine by ^{14}C-orotic acid when the cells were treated with Ara-6-MP. When cell-free extracts from susceptible tumors were assayed for cytidylate reductase, Ara-6-MP, at levels equivalent to the concentration one would have expected to achieve *in vivo* in tissue water with a carcinostatic dose of Ara-6-MP, produced an equivalent inhibition of cytidylate reductase. Therapeutic doses of 30–60 mg/kg would produce, for a time, concentrations of $3–7 \times 10^{-5}$ M Ara-6-MP in body water. A concentration of 5×10^{-5} M Ara-6-MP produced

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[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

immediate inhibition of cytidylate reductase of 25–35% and, after a brief preincubation with drug, of 75–85%.

Two lines of cells sensitive to Ara-6-MP, an L1210 line and an Ehrlich line, produced resistant lines. The relative sensitivities in a limited therapy test are illustrated in table 1. Cell-free extracts were made from cells of each line and tested for sensitivity to Ara-6-MP of their cytidylate reductases. The results are given in table 2. This limited series of cell lines gave a correlation between response and inhibition of the reductase performed on cell-free extracts.

It was found that the reductase was stable indefinitely in tissue samples, mouse or human, stored at —70°C. However, once cell-free extracts were made, they deteriorated in a few days at the same temperature. Extracts of mouse tissues could be preincubated (20 minutes), but preincubation of extracts from human tumors led to inactivation of the reductase, even without the drug addition, *e.g.*, 50% loss at 0°C in 2 hours, and so was not included in our procedure.

TABLE 1.—Therapy tests on mouse tumor lines*

Tumor	Treatment	Survival time (days)
L1210	—	7.6 ± 0.2
	Ara-6-MP	12.1 ± 0.3
Ehrlich	—	9.9 ± 1.3
	Ara-6-MP	16.6 ± 1.8
L1210R	—	7.8 ± 0.3
	Ara-6-MP	7.7 ± 0.4
Ehrlich R	—	10.3 ± 1.5
	Ara-6-MP	9.6 ± 1.8

* Groups of 10 female mice inoculated ip with 5 × 10^5 cells/mouse and treated two times daily on days 1–6 thereafter with saline or saline solutions (*p*H 7.5) of Ara-6-MP at 50 mg/kg.

TABLE 2.—Cytidylate reductase assays on murine tumor cell extracts*

Cell extract	Addition	dCDP† formed (nmoles)	Inhibition (%)
L1210	None	0.70	
	Ara-6-MP, 5 × 10^{-5} M	0.43	39
Ehrlich	None	0.39	
	Ara-6-MP, 5 × 10^{-5} M	0.12	69
L1210 R	None	0.68	
	Ara-6-MP, 5 × 10^{-5} M	0.69	0
Ehrlich R	None	0.42	
	Ara-6-MP, 5 × 10^{-5} M	0.41	2

* Incubation for 20 minutes without preincubation. Medium contained ^{14}C-cytidine diphosphate, 100 nmoles; adenosine triphosphate, 0.5 μmoles; $MgCl_2$, 1.5 μmoles; reduced triphosphopyridine nucleotide, 60 nmoles; Tris buffer at *p*H 7.4, 50 μmoles; cell-free extract equivalent to 2.4 mg protein. Volume 0.60 ml.

† dCDP = deoxycytidine diphosphate.

The assay was conducted on material from 19 human tumors, obtained at surgery and stored at —70°C until assayed. The results, presented in table 3, are expressed on the basis of the DNA content of the tissues because of their varied con-

TABLE 3.—Cytidylate reductase assays on extracts of human tumors*

Tissue	DNA (mg/g)	Cytidylate reductase (nmoles dCDP†) Control	+ Ara-6-MP	Control (%)
Breast carcinoma (D.M.)	0.393	1.06	0.72	*68*
” ” (69–837)	0.225	—	—	
” ” (G.G.)	1.04	1.02	0.78	76
” ” (B.)	0.82	3.04	2.04	*67*
” ” (G.C.)	1.96	3.04	2.34	77
” ” (G.W.)	0.718	1.79	1.73	97
Colon carcinoma (J.)	0.885	—	—	
” ” (C.O.)	4.1	0.16	0.16	114
Cecum carcinoma (L.M.)	2.51	0.61	0.56	92
Seminoma (C.W.)	4.24	0.20	0.16	80
” (S.)	2.19	0.41	0.39	95
Testicular carcinoma (R.)	1.84	0.94	0.82	87
Hepatoma (U.)	1.46	0.15	0.15	100
Squamous cell carcinoma of leg (H.)	0.633	3.19	1.08	*34*
Rectal carcinoma (S.M.)	0.676	0.59	0.27	*46*
Sarcoma of abdominal wall (F.S.)	2.96	0.18	0.11	*61*
Stomach carcinoma (L.J.)	0.56	—	—	
Liposarcoma of leg (D.G.)	0.805	5.40	5.00	92
Malignant melanoma (F.)	1.66	1.62	1.19	73

* Frozen tissue samples were homogenized in 4 volumes of saline with a VirTis 23 for 2 minutes. Homogenates were centrifuged 60 minutes at 18,000 × *g*. The residue was used to determine DNA. The extract was used for reductase assays in the medium described in table 2. Duplicate samples were incubated for 20 minutes, with and without Ara-6-MP added at 5 × 10^{-5} M.

† dCDP = deoxycytidine diphosphate.

tent of fat, stroma, etc. It appears that at least 5 of these samples showed inhibition comparable to that observed for responsive experimental tumor systems. Thus, such an assay on extracts of frozen-stored tumors might permit selection of the sensitive tumors. Three tumor samples provided no assay results. These may have been deteriorated samples not obtained sufficiently rapidly at surgery, but no direct evaluation of this was possible. We hope to extend the series in parallel with clinical trials.

A second example with quite different circumstances involves the nucleoside analogue, arabinosyladenine (Ara-A). Brink and LePage showed that this nucleoside was deaminated by adenosine deaminase and phosphorylated to the ATP analogue by cell kinases *(5–7)*. It was carcinostatic to a series of mouse ascites tumors that had low or moderate levels of adenosine deaminase and ineffective on tumors with high levels of the enzyme. York and LePage *(8)* and Furth and Cohen *(9)* showed that the Ara-ATP inhibited DNA polymerase. This appeared to be the mechanism by which it was exerting its carcinostatic effects. Normal tissues had the protection of their adenosine deaminase, since Ara-hypoxanthine was inactive. Koshiura and LePage *(10)* showed that a spectrum of human tumor samples varied in adenosine deaminase from low to high levels.

When the animal tumor spectrum was extended, the correlation was not as clear. It would be logical for the response of a tumor to such an agent, when both activation and destruction occur, to depend on both rate of activation and rate of destruction. Conceivably, a tumor exhibiting a relatively high rate of catabolism of the drug would be responsive if it also had a high rate of activation. Correspondingly, a tumor with a relatively low catabolic rate might be unresponsive if drug activation was very low. Thus, as a principle, one should determine both catabolism and activation rates for such a drug. Cytosine arabinoside would seem to be a similar example.

Without reiterating the details of the therapy of the animal tumor spectrum, table 4 shows the assays for deamination and phosphorylation of Ara-A in some murine tumors. Also included are some similar assays for a series of 12 human tumor biopsies. The data in table 4 show: *a)* that there are considerable variations in both deaminase and kinase activities between murine tumors; *b)* that the correlation would, as expected, improve when both factors were taken into consideration; and *c)* that the limited series of human tumor samples examined also showed such variations. One can observe examples where either assay alone would give a different evaluation than the two taken together. These are tests, of course, that lend

TABLE 4.—Adenosine deaminase and kinase assays on tumor samples*

Tissue		Response	Deamination	Nucleotide formation	Ratio
Dunning leukemia	(rat)	—	23	0.11	209
Walker 256	(rat)	—	63	0.21	300
6C3HED lymphosarcoma	(mouse)	++++	4	0.57	7
TA3 carcinoma	(mouse)	+++	26	0.54	48
L1210 leukemia	(mouse)	—	100	0.28	357
Lewis lung carcinoma	(mouse)	++	24	0.76	32
Sarcoma 180	(mouse)	+	66	0.65	101
Carcinoma 1025	(mouse)	++	24	0.63	38
Ridgeway osteosarcoma	(mouse)	++	36	0.83	43
Ehrlich carcinoma	(mouse)	++	29	0.19	152
Human					
Breast carcinoma	(M.)		15	0.57	26
" "	(G.G)		6	0.20	30
" "	(G.B.)		105	0.91	115
" "	(G.C.)		9	3.82	2.3
" "	(W.)		30	0.67	45
Liposarcoma	(D.G.)		16	1.72	9.3
Seminoma	(S.)		18	0.52	35
"	(C.W.)		38	0.13	292
Colon carcinoma	(J.)		38	0.16	62
" "	(L.M.)		89	0.28	318
Malignant melanoma	(F.)		41	0.33	124

* The figures for mouse and rat tissues are expressed as μmoles substrate converted/hr/g wet weight. Those for human tissues are expressed as μmoles/hr/mg DNA.

themselves to evaluations of frozen-stored samples of solid tumors. Where cell suspensions of malignant white blood cells or marrow samples are available, it would perhaps be desirable to monitor the inhibition of utilization of a labeled DNA precursor rather than this indirect approach, but such a direct approach would not be as feasible for the solid tumor samples.

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Biochemical Approaches to Prediction of Response in Solid Tumors[1, 2, 3]

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THERE IS no proved method by which one may select the best drug for treating cancer in a specific patient. Development of a means for predicting drug sensitivity before therapy will avoid the trial-and-error method of drug selection. Currently, the selection of drugs is based on one's prior experience with histologically similar lesions. However, response to therapy seems only loosely related to histologic features. Electron microscopic *(1),* histochemical *(2),* and biochemical *(3–5)* studies indicate that similarly appearing tumors may differ both morphologically and biochemically. Studies of Kissel *et al. (6, 7)* confirmed that metabolic differences exist *in vivo.*

Many attempts have been made to develop a method for predicting tumor sensitivity. Hirschberg *(8),* in a rather pessimistic article, listed a number of factors, such as drug excretion and alteration, cumulative toxicity, tumor-host interaction, and drug distribution, which are important in the whole animal but are not evaluated in *in vitro* systems. Such factors may have caused the discrepancies demonstrated by Eagle and Foley *(9)* between sensitivity in tissue culture and drug response in the whole animal. Generally, drugs effective against transplantable tumors *in vitro* were also found to be effective *in vivo.* However, of 180 compounds tested, one-fifth of the drugs active *in vivo* failed *in vitro* and one-fifth of the drugs active *in vitro* were inactive *in vivo.* Wright *et al. (10)* related the histologic cytoxicity produced by drugs in tissue culture with response of the same human tumors to chemotherapy. Thirty-five percent of the specimens could not be grown in tissue culture, but 180 were grown and could be studied. In these, clinical response to chemotherapy correlated with the *in vitro* findings in one-half of the cases, equivocally correlated in one-fourth, and was not correlated in one-fourth.

Drug effect on metabolic activity of human tumors *in vitro* was studied by means of inhibition of dehydrogenase activity *(11–13)* and by changes in tumor respiration and protein and nucleic acid metabolism *(14, 15).* Black and Speer *(11)* correlated the *in vitro* response of human tumors to triethylene melamine (TEM) and to the combination of TEM and urethan with subsequent clinical response. If one assumes greater than 30% *in vitro* inhibition to be significant, the correlation was quite good, with 5% false positive values and 13% false negative values. A different system was used by DiPaolo and Dowd *(12)* to measure dehydrogenase activity. They reported 42% false positives and no false negatives. Using a similar system, Kondo *et al. (13)* reported 18 cases, 11 of which responded *in vitro* and *in vivo.* Four cases responded neither *in vitro* nor *in vivo,* and three cases responded only *in vitro.* Sky-Peck *et al. (4)* studied the effect of drugs on various biochemical parameters of human tumors. In breast carcinoma, it was found that rapid progression of the disease occurred when estradiol produced an increase in formate incorporation into protein. If estradiol caused a decrease in formate incorporation, clinical improvement occurred. Sky-Peck *(14)* incubated human tumor bioposies taken before and after cancer chemotherapy with tritiated thymidine and reported an 88% correlation between changes occurring in uptake and the clinical course of the disease. Bickis and Henderson *(15, 16)* studied respiration, glycolysis, and precursor incorporation into protein and nucleic acids in a number of human tumors. Interesting correlations were demonstrated between *in vitro* effects produced by drugs and their clinical effectiveness.

In experimental systems a number of studies with the fluorinated pyrimidines support the con-

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[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

[3] Supported by Public Health Service grant CA 05953 from the National Cancer Institute.

[4] I acknowledge the valuable technical assistance of Dr. Kiyoko Watanabe, Miss Marilyn Johnson, and Mrs. Phyllis Walker.

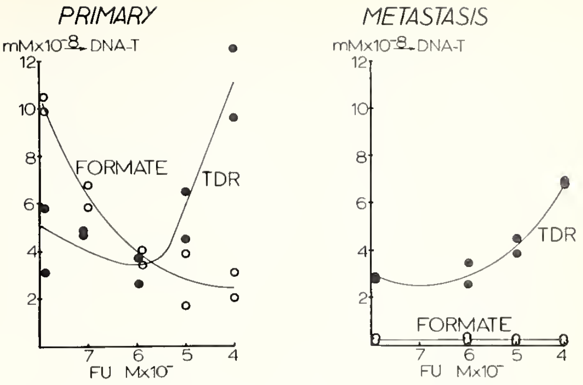
cept of biochemical determinants for tumor sensitivity. Reichard. Sköld, *et al.* *(17–19)* reported the development of four 5-fluorouracil (FU)-resistant lines of Ehrlich ascites tumor in which the development of resistance was associated with a decrease in uridine kinase activity. Kessel *et al.* reported similar findings *(20)*. Uridine kinase converts uridine to uridine-5′-monophosphate and 5-fluorouridine to 5-fluorouridine-5′monophosphate (FURP). This ribotide must then be reduced or the ribose-5′-phosphate exchanged to produce the active form of the drug. 5-fluoro 2′-deoxyuridine-5′-monophosphate (FUDRP). Kasbekar and Greenberg *(21, 22)* demonstrated that orotidine-5-pyrophosporylase converts FU to FURP and that development of FU resistance in a strain of Gardner lymphosarcoma was associated with the loss of this enzyme. They also showed that FU inhibited orotic acid decarboxylation in calf thymus. Hartmann, Heidelberger, *et al.* *(23, 24)* found that resistance developed in Ehrlich ascites carcinoma when thymidylate synthetase became insensitive to FUDRP. Morris and Fischer *(25, 26)* and Morse, Gentry, *et al.* *(27–29)* demonstrated resistance due to a deficiency of thymidine kinase which converts 5-fluoro-2′-deoxyuridine (FUDR) to FUDRP. Heidelberger *et al.* found differences in the degree to which fluorinated pyrimidines inhibited incorporation of formate into DNA-thymine in sensitive and resistant strains of Ehrlich ascites carcinoma cells *(24, 30)*. Comparable results were obtained *in vivo* and *in vitro*. Attempts to apply this type of information to the development of a predicting system for fluorinated pyrimidine sensitivity in human tumors were reported by Birnie and Heidelberger *(5)*. They studied the effect of fluorinated pyrimidines on the incorporation of ^{14}C-formate into acid-soluble thymine in human tumors. A marked variation in the uptake in various tumors and a variable effect were produced on this uptake by the fluorinated pyrimidines.

In my early studies, I used various tritiated pyrimidines as precursors, and the effect of the fluorinated pyrimidines on their uptake by human tumors was evaluated by autoradiography *(31, 32)*. The result of these studies will be subsequently discussed.

First, however, consider what is expected of a predicting system for chemotherapy. Drugs that are made available for clinical trials have demonstrated antitumor activity in various animal screening systems. In such systems the ability of drugs to retard the rate of growth of tumors or to prolong host survival is evaluated. Because such baselines are obviously not available to the clinician, the only accepted criterion for response in human cancer is 50% regression in the size of a measurable lesion. This is a much more stringent requirement for response than is required in experimental systems. Remarkably, by these criteria some responses are seen in human tumors. It appears, therefore, that many human tumors may be more sensitive than the tumors used in animal test systems. Regression in tumor size requires that dead cells be removed from the tumor at a rate which exceeds the rate of overgrowth by drug-resistant tumor cells. Therefore, possibly drugs kill many more cancer cells than we realize. Evaluation of tumor cell kill appears to be one of the chief problems in chemotherapy in general and in evaluating predicting systems in specific. It is possible that predicting systems are more sensitive and accurate in assessing human tumor cell sensitivity than we can recognize clinically.

Because of the relative clinical effectiveness of the fluorinated pyrimidines and their extensive use at our institution, my studies have chiefly concerned these drugs. Multiple drugs have not been studied because it appeared desirable to establish a relationship between *in vitro* and *in vivo* tumor response with one drug before attempting to evaluate a number of drugs. At the time the first studies were undertaken, it was not established that basic biochemical factors of tumor cell metabolism were important in determining drug responsiveness in human tumors. In addition, very little was known either about the biochemical activity of neoplastic and non-neoplastic constituents of human tumors or their relative behavior in tissue culture. For this reason, I chose to incubate human tumor slices in a short-term tissue culture system containing various tritiated pyrimidines for precursors and used autoradiography as the method for assessing uptake and drug effect. In general it was found that the malignant elements and only the malignant components of human tumors incorporated these precursors into their DNA. Tremendous differences were found both in baseline incorporation of the compounds (fig. 1) and in their response to the fluorinated pyrimidines. In 114 patients studied, it appeared that the metabolic behavior of tumors as determined *in vitro* was related to tumor response in the patients *(24)*. These data established the validity of such an *in vitro* system in studying drug effect on human tumors. The method of tissue preparation for autoradiography limited the number of precursors that could be studied, sampling problems existed, and interpretation of autoradiographs was both time consuming and somewhat subjective. Because of this, we changed to a biochemical method of assay based on the method described by Birnie and Heidelberger *(5)*. Rather than study acid-soluble thymine synthesis, we elected to study the synthesis of DNA-thymine in these tumors.

We now isolate the DNA-thymine from human tumors after incubation with ^{3}H-methyl-thymidine and ^{14}C-3-serine or ^{14}C-formate. Simultaneous evaluation of DNA-thymine synthesis by alternative pathways is therefore possible. A detailed account of this work has been published *(33)*. In human tumors, a drug-induced decrease in utilization of formate or serine as a source of the 5-methyl group of DNA-thymine is almost always accompanied by a compensatory increase in thymidine incorporation. This provides the tumor with a mechanism for bypassing the block of FU. The most clinically sensitive human tumors studied with autoradiography, however, demonstrated a decrease in thymidine uptake in the presence of the fluorinated pyrimidines (table 1). In many instances, there was little metabolic similarity between the primary tumor and its metastases (text-fig. 1). This is consistent with the mixed response frequently seen during drug therapy.



TEXT-FIGURE 1.—Adenocarcinoma of the descending colon with hepatic metastases. The *abscissa* represents the molar concentration of 5-FU present in the medium during a 24-hour tissue culture incubation. ^{3}H-methyl-thymidine and ^{14}C-formate were also added to the medium (final molarities: TDR $= 1.1 \times 10^{-7}$; formate $= 4 \times 10^{-6}$). The *ordinate* gives the amount of each precursor incorporated into the DNA-thymine of 1 ml of tumor cells in primary and metastatic tumor. *Note* difference in uptake pattern of the 2 tumors. Hepatic metastases did not respond clinically to intrahepatic artery infusion with FU.

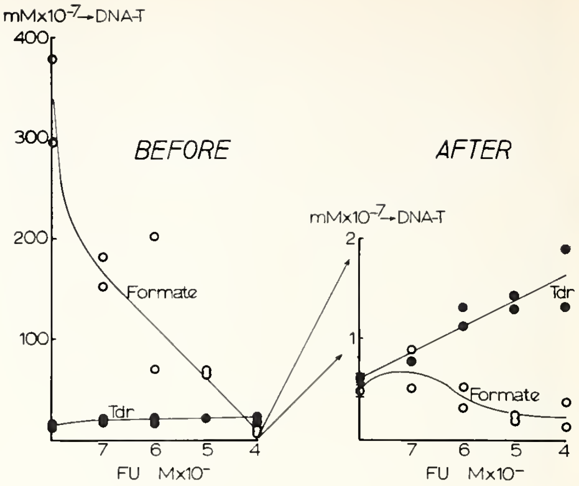
Clinical data regarding tumor response to FU were available for 15 patients in whom the relationship between clinical response and *in vitro* inhibition of formate incorporation into DNA-thymine produced by FU is shown in text-figure 2. A colon carcinoma (text-fig. 1) was surgically resected, and hepatic artery infusion was done for hepatic metastases. Clinical progression of the metastases was apparent after 6 months of therapy. In this tumor, the formate uptake differed markedly

TABLE 1.—Effect of drug on nucleoside incorporation—patient response*

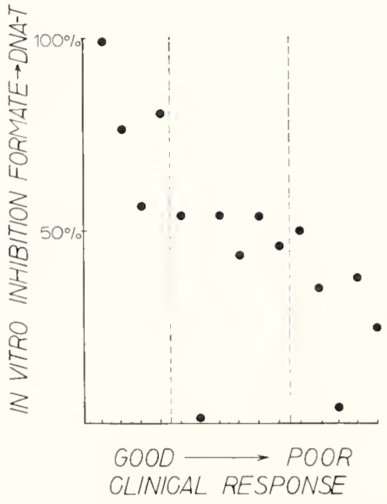
Pattern	Thymidine	Uridine → DNA	Number of responders	Number of nonresponders
Insensitive	(No uptake)	(No uptake)	0	29
	(No uptake)	No change	0	1
	No change	(No uptake)	0	15
	No change	No change	0	9
	Increase	No change	0	7
Sensitive	Decrease	Decrease	4	0
	Decrease	No change	1	0
	Decrease	(No uptake)	6	0
	No change	Decrease	2	1
Indeterminate	Increase	Decrease	9	8
	Increase	No uptake	7	16

* The data presented in table 1 have been grouped according to the degree of correlation between the various *in vitro* uptake patterns and patient response to therapy. Clinical class 2 and 3 have been combined as "responders" and clinical class 1 represented the nonresponders (*10*). There was extremely good correlation in the insensitive and sensitive patterns. The response rate was high in the indeterminate pattern, but the nonresponders and responders could not be distinguished on the basis of their *in vitro* uptake patterns.

in the primary tumor and its hepatic metastases. The primary tumor had a significant uptake of formate into DNA-thymine, and this uptake was quite sensitive to 5-FU. The hepatic metastases, on the other hand, showed little formate uptake, and it was unaffected by 5-FU. The tumor from both sites incorporated substantial amounts of thymidine into DNA-thymine, and the incorporation increased markedly with increasing concentrations of 5-FU. Clinical sensitivity to FU was found in 2 tumors. A bronchogenic carcinoma (text-fig. 3) had metastases to the supraclavicular area and chest wall. Excised tumor showed a marked decrease of formate incorporation proportional to the concentration of FU in the medium. Marked clinical regression was maintained with therapy for 3 months before resistance became apparent. The surgically excised resistant tumor incorporated much less formate than was found before therapy, and the residual formate incorporation was insignificantly affected by FU.



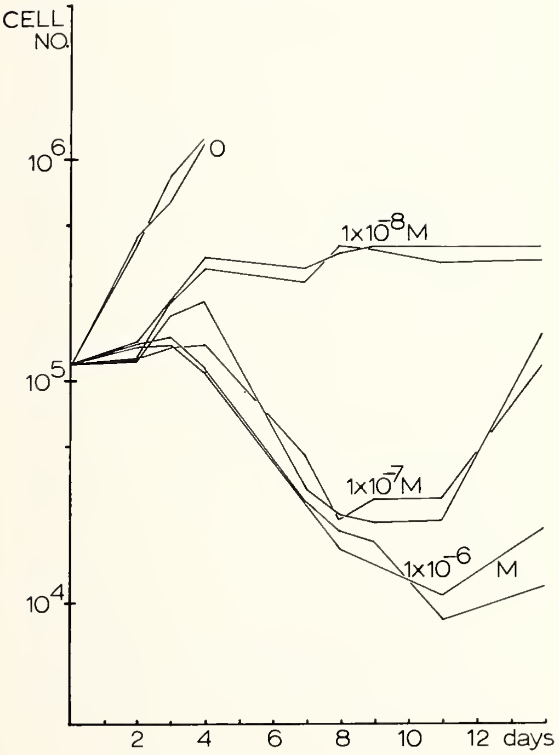
Text-figure 3.—Squamous cell carcinoma of lung had supraclavicular metastases. Incubation conditions were the same as outline in text-figure 1. The tumor was biopsied and studied before FU systemic therapy was started ("Before" *above*). A good clinical remission, maintained for 3 months, was obtained. The rapidly growing resistant tumor was excised and studied ("After" *above*). *Note* that the "After" ordinate was multiplied by 100. The formate uptake and its sensitivity to FU were profoundly altered by the clinical chemotherapy. DNA-T = DNA-thymine.



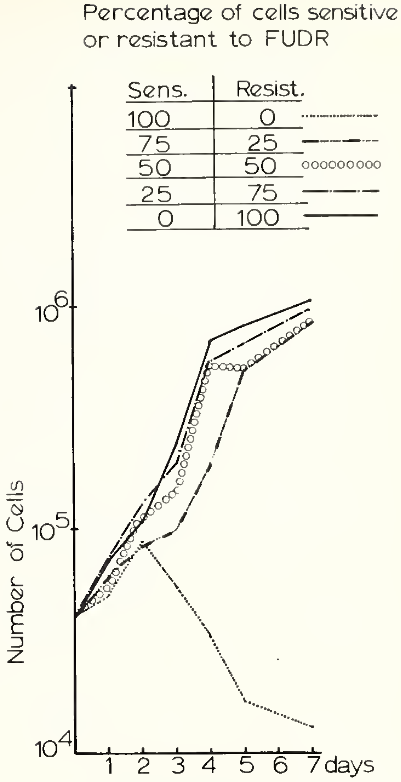
Text-figure 2.—The clinical response of 15 patients was ranked according to effectiveness of FU therapy. The response was correlated with the degree of inhibition of incorporation of formate into DNA-thymine produced by FU *in vitro*.

This last case clearly demonstrates that the tumor was biochemically changed by drug treatment. We conducted a number of studies with Novikoff hepatoma in tissue culture to determine whether tumor resistance develops due to drug-induced changes or whether resistance develops from overgrowth by a small proportion of initially present resistant cells. From the original FUDR-sensitive strain, Morse and Potter *(27)* developed a strain resistant to 1×10^{-5} M FUDR by serial transfer to media containing increasing FUDR concentrations. They showed that resistance in this strain was due to the lack of thymidine kinase. The cells were therefore unable to convert FUDR to the biologically active monophosphate. We found that resistance developed in the originally sensitive culture at a rate that depended on the concentration of FUDR in the medium (text-fig. 4). Cloning studies demonstrated that the originally sensitive cell line contained cells that were resistant to FUDR and that fewer cells were resistant at higher FUDR concentrations (table 2). Studies with mixtures of resistant and sensitive cells demonstrated that as many as 75% of the cells may be killed and the effect scarcely noticeable on growth curves (text-fig. 5). This study confirmed that populations of tumor cells have a wide degree of variation in drug sensitivity and that a certain proportion of cells in a tumor are not sensitive to a particular concentration of drug. Although a significant cell kill may be produced by a specific drug, the resistant cells become dominant and their biochemical characteristics are found in the tumor after development of drug resistance.

From these data, it can be concluded that there are definable biochemical factors present in varying degrees in tumor cells. Some of these factors determine response to drug therapy. This was determined in experimental systems, and data are accumulating which indicate that the same is true for human tumors. *In vitro* response to drug is related to drug concentration; so, undoubtedly, the concentration of drug reaching the tumor is also an important factor in response. Obviously, there is a great deal that we do not know about determinants of drug sensitivity in human tumors. We are greatly hampered by our inability to clinically detect extensive cell kill in some tumors. As some of these problems are defined and solved, we will move closer to our goal of developing a true predicting system for tumor sensitivity.



Text-figure 4.—Growth curves for sensitive Novikoff hepatoma in the presence of various concentrations of FUDR.



Text-figure 5.—Growth curves for various proportions of sensitive and resistant Novikoff hepatoma incubated in medium containing 1 × 10^{-5} M FUDR.

Table 2.—Effect of FUDR concentration on colony formation by Novikoff hepatoma cells*

Concentrations of FUDR (M)	Number of colonies	Percent of control
0	45,000	
1 × 10^{-8}	2,650	5.9
1 × 10^{-7}	1,740	3.9
1 × 10^{-6}	1,340	3.0
1 × 10^{-5}	270	0.6

* Colony-forming ability of sensitive Novikoff hepatoma in medium containing various concentrations of FUDR. Original inoculum was 500,000 cells. Aggregates containing more than 10 cells 4 days after inoculation were considered to be colonies.

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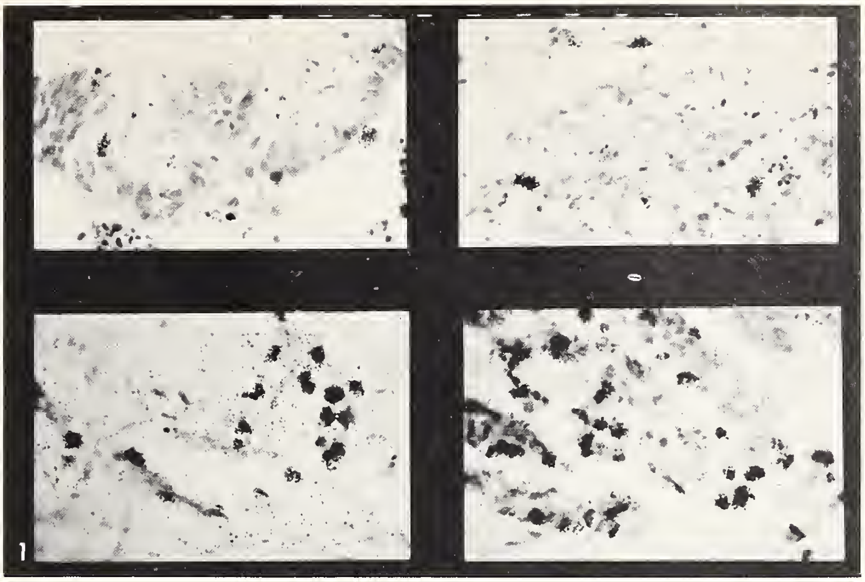

FIGURE 1.—Four different but histologically similar colon carcinomas were incubated for 24 hours with 1 μc/ml of thymidine-methyl (specific activity = 12 Ci/mmole). Acid solubles were removed and autoradiographs were made. A great difference can be noted in the amount of thymidine incorporated by the various tumors.

Effects of Chemotherapy on the Incorporation of ^{3}H-Thymidine Into DNA of Human Neoplastic Tissue[1, 2]

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PREVIOUS STUDIES *(1–3)* of the *in vitro* incorporation of ^{14}C-formate into normal and neoplastic tumor tissues before and after the initiation of chemotherapy indicated a correlation between the uptake of ^{14}C-formate into protein-bound serine and the clinical course of the disease. However, due to the heterogeneity of tissues and the complexity of interpretation of biochemical changes expressed as relative specific activity, it was believed that a more direct and more specific precursor should be used to correlate tumor metabolism with therapeutic response. Thymidine, a pyrimidine deoxynucleotide, is incorporated exclusively into DNA during a limited period of interphase *(4, 5)* and, with few exceptions, only in those cells preparing for division. Therefore, in a given cell population, the incidence of cells labeled by tritiated thymidine should be proportional to the rate at which cells proliferate. Furthermore, because the period of DNA synthesis is considerably longer than the period required for mitosis, the DNA synthesis index should be a more sensitive indicator of renewal rate of cells than the mitotic index *(6)*. Consequently, in any population of cells, the specific activity of DNA or the percent cell nuclei labeled depend on the population of cells synthesizing DNA at the time label is administered.

For any chemotherapeutic agent to be effective in the treatment of cancer, it should decrease cell multiplication and thus DNA synthesis, regardless of its mechanism of action. Therefore, a comparison between ^{3}H-thymidine (^{3}H-TDR) incorporation into tumor nuclei, before and after the initiation of therapy, and the clinical effectiveness of the therapeutic agent should be of value as a means of screening prospective chemotherapeutic agents in humans. Previous results from this laboratory *(7)* have caused us to examine the validity of using quantitative changes in DNA synthesis *in vitro* as an indicator of cytotoxicity in Ehrlich ascites and in Sarcoma 180 tumors exposed *in vivo* to known antitumor drugs. One group of drugs previously shown to be carcinostatic for these tumors produced a significant decrease in DNA synthesis, whereas a second group of drugs, previously shown not to be carcinostatic, produced no significant decrease in DNA synthesis.

However, many compounds which have shown significant carcinostatic activity, when screened in animal tumors or in tissue culture, have shown very little effect, if any, when used in the treatment of human cancer. The development of any technique that can demonstrate changes in human tumor due to therapy would greatly aid and speed up the human cancer chemotherapy program.

The present study was undertaken to investigate the feasibility of comparing changes in the *in vitro* incorporation of ^{3}H-TDR into human neoplastic nuclear DNA produced by administration of drugs to the patient and to correlate these results with the clinical response to therapy.

MATERIALS AND METHODS

The selection of patients and therapy was at the discretion of the chemotherapists. In many instances, double-blind studies were undertaken, and the identity of the specific compound administered was not revealed until the end of the chemotherapy study. Therapy was not discontinued at the time of the second biopsy, as the preliminary program of evaluation of this technique of tumor assay was designed not to interfere with the clinical chemotherapy programs. Therefore, at the conclusion of therapy or at a time when the patient's response could be determined, the data on the uptake of ^{3}H-TDR were evaluated.

Patients with disseminated cancer, who had pro-

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[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

gressive disease and had no clinical evidence of effects from previous therapy, were selected for this study. Normal and neoplastic human tissues were obtained from the same individual by biopsy before, and at various intervals after, the initiation of chemotherapy.

The tissues, weighing approximately 0.5 g, were immediately cut with a Stadie-Riggs slicer and placed in 3 ml of Krebs-Ringer bicarbonate medium containing 5 μc ^{3}H-TDR (360 μc/μM). The tissues were shaken while being incubated at 37°C in an atmosphere of 95% oxygen and 5% carbon dioxide for 30 minutes *(8)*. The incubations were terminated by placing a portion of the tissues in warm formalin before preparation of autoradiographs. The remaining tissues were placed in 5% trichloroacetic acid (TCA) prior to the isolation and determination of DNA and free thymidine.

The tissues fixed in formalin were washed in cold water overnight to remove remaining free thymidine. They were then mounted in paraffin and sectioned for the preparation of autoradiographs with the use of Kodak liquid NTB$_3$ emulsion. Three weeks' exposure to the tritium-labeled tissues was usually required to develop sufficient granules over the cell nuclei. After photographic development, the slides were stained with Harris' hematoxylin and eosin. The autoradiographs were subjected to cell counts and the results expressed as:

$$\frac{\text{Number of tumor nuclei with grains}}{\text{Total No. of tumor nuclei counted}} \times 100.$$

A minimum of 3,000 cells was counted in 6 microscopic fields on each slide. In some instances, the average grain count in labeled nuclei was determined for comparison with the specific activity measurements.

The tissues fixed with TCA were washed 3 times in cold 5% TCA and defatted before extraction with hot TCA to obtain the nucleic acid constituents. Tritium, representing DNA-thymine, was estimated by scintillation counting, while the quantity of DNA was determined on a sample of the same fraction by its reaction to diphenylamine *(9)*. The results are expressed as:

$$\frac{\text{cpm/mg DNA}}{\text{cpm}/\mu\text{M}\ ^3\text{H-TDR}}.$$

Changes in the thymidine pool, expressed as specific activity, were determined on tissues homogenized in nonlabeled medium by addition of 1 μc of ^{3}H-TDR (2.5×10^{-3} μM) to the cold TCA extract. The mixture of endogenous and exogenous thymidine was isolated by chromatography on Whatman AE81 filter paper saturated with 0.35 M ammonium formate, *p*H 5.5, by the method of Bukovsky and Roth *(10)*, ammonia-isobutyric acid according to Fink *et al. (11)*, and ethyl acetate-formic acid system according to Smith *(12)*. The thymidine was eluted with 0.1 N HCl, and its specific activity estimated by ultraviolet absorption at 260 mμ and liquid scintillation counting. Chromatography was repeated to obtain a constant specific activity.

Occasionally it was possible to give intravenous injections of ^{3}H-TDR (1 mc/10 kg body wt) into patients with advanced metastatic disease. Thymidine injected in this manner was utilized in approximately 30 minutes, resulting in pulse labeling of DNA. By obtaining tissue samples at various intervals after the initial labeling, it was possible to get an estimate of the length of the cell cycle for both normal and neoplastic tissues. Autoradiographic techniques, similar to those developed by Howard and Pelc *(13)*, Lesher *et al. (14)* and Baserga *(15)*, were used to study the life cycle of cells in human tissues. In some instances, in which it was impossible to obtain multiple samples over a long period, double-labeling experiments were done. Tritiated thymidine was used *in vivo,* followed by 30-minute incubations with ^{14}C-thymidine *in vitro* by the method of Bresciani *(16)*. The preliminary data were obtained in this manner, and they help to confirm previous results, *i.e.,* that human tumor cells do not necessarily proliferate faster than normal cells.

RESULTS AND DISCUSSION

During the past 5 years, 474 biopsies have been performed on 208 patients with various forms of metastatic cancer. Of these, 27 patients had no subsequent biopsy after the initial one, 7 patients died before the second biopsy, 21 patients had subsequent biopsies of tissues which were not comparable, and 18 patients could not be clinically evaluated. The data from the remaining 135 patients could be evaluated and are presented here.

Despite the inherent problems of tissue heterogeneity and tissue sampling, there have been fairly good correlations between the changes in percent of cells labeled, relative specific activity, or average grain count per labeled cell and the effects of chemotherapy. Data presented in tables 1 and 2 show the percent tumor cells labeled before and 7 days after the initiation of therapy, compared with the eventual therapeutic results. In almost every instance, the percent uptake of the ^{3}H-TDR into cells changed as a result of therapy. These changes, which had occurred within a 7-day period, correlated surprisingly well with the clinical effects of therapy, evaluated after a period of 3 months. In fact, there was an 87% correlation between the uptake of ^{3}H-TDR into tumor DNA and the clinical course of the disease. This correlation was verified by a Wilcoxon Ranked-Sum Test, where the criteria for determining positive or negative correlations were based on changes of 50% or greater. In general, the results indicate that an *in vivo–in vitro* testing system, in which the patient received a test dose of the chemotherapeutic agent and a specimen of the tumor was removed for incubation *in vitro*, is an efficient method of evaluating drug toxicity or therapy in human tumors.

The results obtained with human tumors substantiated results obtained previously by Wolberg and Brown *(17)*, who studied the *in vitro* incorporation of uridine and thymidine into human tumors, and in previously reported experiments with animal tumors *(7)*. However, this technique of measuring chemotherapeutic effectiveness by autoradiography appears only practical as a retrospective study in a large population, due to the time needed to complete an evaluation of an individual. The direct measurement of relative specific activity of DNA may be used, however, with less accuracy due to tissue heterogeneity and the inability to see the tissues being measured. However, this more rapid technique may be used if alternate slices of tumor are compared for metabolism and histology.

Despite the fact that specific activity measurements are inherently less reliable than autoradiographic techniques in a heterogeneous system, specific activity measurements appear to correlate sufficiently to be considered as a possible assay procedure. Although the statistical analysis is incomplete due to the small population, there appears to be approximately an 80% chance of determining at the end of the 1st week of therapy whether or not the patient is responding to therapy.

Previous investigators *(7)* showed that a single observation of drug effect on DNA synthesis may

TABLE 1.—Effects of chemotherapy on the incorporation of ^{3}H-TDR into the DNA of human neoplastic tissues

Disease	Therapy	Clinical result*	Percent cells labeled	
			Before	After
Adenocarcinoma of the bowel	5-FUDR†	Progression	9.17 ± 2.1	29.80 ± 3.4
Cancer of the colon	”	Progression	12.75 ± 3.2	19.49 ± 4.6
Cancer of the colon	”	Progression	10.10 ± 3.6	1.41 ± 0.56‡
Cancer of the colon	”	Regression	17.39 ± 5.1	1.81 ± 0.71
Cancer of the colon	”	Progression	12.66 ± 3.4	9.56 ± 3.7
Cancer of the rectum	”	Static	5.16 ± 2.1	1.46 ± 0.67
Hepatoma	”	Progression§	5.40 ± 1.2	5.62 ± 1.4
Cancer of the breast	”	Progression	0.65 ± 0.12	5.58 ± 2.2
Cancer of the breast	”	Progression	1.46 ± 0.42	5.87 ± 1.4
Cancer of the breast	”	Regression	3.98 ± 1.0	0.44 ± 0.13
Cancer of the breast	”	Regression	4.26 ± 0.64	1.41 ± 0.41
Cancer of the breast	”	Progression§	0.51 ± 0.17	0.61 ± 0.23
Melanoma	Vincristine	Progression	1.39 ± 0.11	6.45 ± 1.45
Melanoma	”	Progression	2.76 ± 1.3	0.71 ± 0.15‡
Cancer of the larynx	”	Static	2.26 ± 0.50	0.66 ± 0.42
Hodgkin's disease	”	Regression	3.37 ± 1.4	0.52 ± 0.11
Lymphoma	”	Progression	1.41 ± 1.0	3.82 ± 1.2
Cancer of the rectum	Hydroxyurea	Progression	0.86 ± 0.41	3.09 ± 1.6
Cancer of the rectum	”	Progression	0.91 ± 0.23	1.72 ± 0.19
Lymphoma	”	Progression	0.48 ± 0.40	5.16 ± 1.3
Sarcoma	Cytoxan	Regression	3.04 ± 1.2	0.72 ± 0.11
Cancer of the ovary	”	Regression	0.59 ± 0.11	0.12 ± 0.04
Cancer of the breast	”	Progression	1.02 ± 0.43	1.87 ± 0.37
Melanoma	Actinomycin D	Progression	1.99 ± 0.34	6.49 ± 1.44
Cancer of the breast	”	Progression	1.39 ± 0.21	5.25 ± 0.82

* All patients had progressive disease at start of therapy.

† 5-Fluorodeoxyuridine.

‡ Inconsistent with clinical interpretations.

§ No clinical response to therapy observed.

TABLE 2.—Effects of hormonal alterations on the incorporation of ^{3}H-TDR into the DNA of human breast cancer

Therapy	Clinical result[*]	Percent cells labeled	
		Before	After
Diethylstilbestrol	Progression	0.96 ± 0.27	13.0 ± 4.0
Diethylstilbestrol	Progression	0.76 ± 0.36	11.4 ± 1.2
Diethylstilbestrol	Progression	0.53 ± 0.22	1.01 ± 0.56
Diethylstilbestrol	Progression	0.54 ± 0.21	0.12 ± 0.08[†]
Diethylstilbestrol	Static	4.01 ± 2.1	1.02 ± 0.49
Estradiol	Progression	0.17 ± 0.06	0.81 ± 0.24
Estradiol	Regression	0.75 ± 0.12	0.16 ± 0.08
Testosterone	Static	0.36 ± 0.11	0.10 ± 0.10
Testosterone	Progression	0.71 ± 4.92	0.30 ± 1.4[†]
2-α-Methyltestosterone	Static	2.30 ± 1.1	0.35 ± 0.1
2-α-Methyltestosterone	Progression	0.35 ± 0.06	1.97 ± 0.21
4-Oxa-5-dihydro methyltestosterone	Regression	0.79 ± 0.14	0.17 ± 0.08
4-Oxa-5-dihydro methyltestosterone	Progression[‡]	0.34 ± 0.14	0.42 ± 0.13
4-Oxa-5-dihydro methyltestosterone	Progression	0.64 ± 0.28	1.81 ± 0.41
X-ray castration	Regression	11.4 ± 1.5	0.23 ± 0.24
Adrenalectomy	Progression	0.23 ± 0.18	0.75 ± 0.21

[*] All patients had progressive disease at start of therapy.

[†] Inconsistent with clinical interpretations.

[‡] No clinical response to therapy observed.

not be accurate. Each group of compounds studied appeared to affect DNA in an individual manner when the parameters of time and magnitude were examined. For example, a single injection of actinomycin D (AD) significantly depressed DNA synthesis 1 hour after injection; 2 hours after injection, synthesis rose 50% above the control value and remained elevated until it again became depressed at 48 hours. A similar result has been shown to follow radiation therapy (table 3), administration of 5-fluorouracil (5-FU), or steroid therapy, but with each being displaced in time. 5-Fluorodeoxyuridine (5-FUDR) produces an apparent initial stimulation of DNA synthesis prior to an inhibition noted after 48 hours.

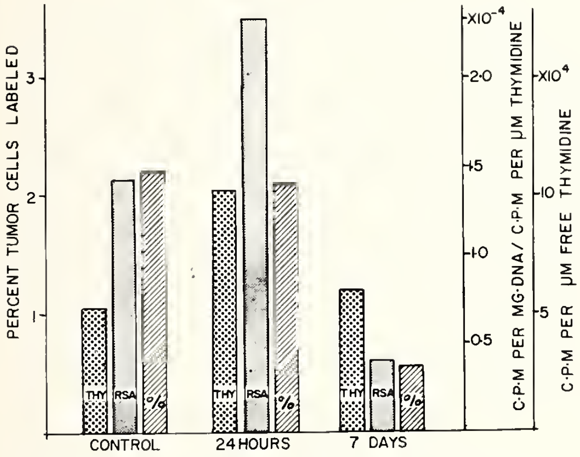
Text-figure 1 shows the effect of 5-FUDR on incorporation of ^{3}H-TDR into tumor DNA. It appears that this apparent initial stimulation of DNA synthesis by 5-FUDR, as measured by specific activity, may be attributed to a decreased thymidine nucleotide pool produced by the inhibition. Thus adding exogenous ^{3}H-TDR resulted in a nucleotide pool of higher specific activity. The effect of 5-FUDR is also illustrated in text-figure 2, where there is an apparent stimulation of specific activity and grain count with a concomitant increase in the specific activity of free thymidine in the incubation mixture. On the other hand, studies on the effects of diethylstilbestrol, estradiol, and X-ray therapy have shown no changes in the specific activity of free thymidine accompanying marked changes in ^{3}H-TDR incorporation into DNA. These changes are illustrated in text-figures 2 and 3. Also, 5-FUDR does not produce an initial stimulation of the percent cells labeled, in contrast to the initial stimulation with hormone or radiation therapy. Radiation appears to produce partial synchrony of a random tumor population, whereas steroid hormones, in hormonally dependent tumors, may actually stimulate DNA synthesis by an effect on DNA polymerase. 5-FU, AD, and many other nucleic acid inhibitors appear to depress the pool

TABLE 3.—Effects of X ray on incorporation of ^{3}H-TDR into DNA from human neoplastic tissues

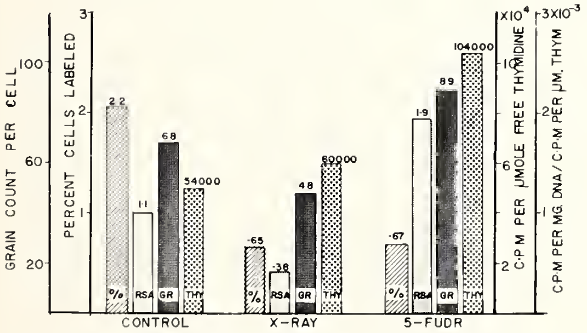
Diagnosis	Label control (%)	Percent label after 300 rads					Estimated generation time (days)
		1 day	2 days	3 days	4 days	5 days	
Hodgkin's disease	17.0	8.5	8.0	5.5	4.7	—	1.5
Adenocarcinoma of breast	6.3	—	2.9	1.9	—	2.3	4.5
Adenocarcinoma of breast	2.9	1.4	2.6	3.5	4.8	—	8.5
Squamous cell carcinoma	2.44	1.8	1.5	—	3.7	3.9	11.0
Squamous cell carcinoma	7.9	3.4	3.8	5.6	3.8	4.3	3.0
Squamous cell carcinoma	9.1	2.7	3.4	4.9	10.4	1.9	2.5
Adenocarcinoma of bowel	22.5	10.8	—	—	7.2	—	1.0
Melanoma	3.1	0.6	3.5	5.0	3.0	—	8.5

size of various precursors leading to DNA synthesis. Therefore, if one wishes to use changes in DNA synthesis as a measure of cytotoxicity, apparently one must establish the individual effects on DNA produced by each antitumor compound. The selection of the 7-day interval used in the present study was found to be the most convenient and accurate for interpretation of results with human tumor tissues.

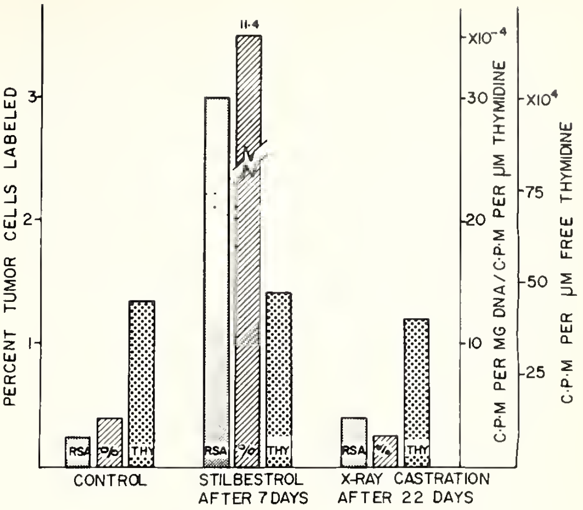
There is good correlation between the percent of cell nuclei labeled with ^{3}H-TDR and the histologic grading or degree of malignancy in human bladder cancer. Text-figure 4 shows results obtained by autoradiographic technique on normal and malignant bladder tissues. Normal bladder has an average of 0.3% labeled nuclei, with an upper range of normal below 1.0%. Normal-appearing mucosa from tumor-bearing patients had a similar average uptake. The papillomas studied averaged 1.7% labeled nuclei. The grade 4 carcinomas averaged close to 10% labeling, and grade 2 and 3 tumors showed intermediate degrees of label.



TEXT-FIGURE 1.—Effects of 5-FUDR on the incorporation of ^{3}H-TDR into the DNA from human neoplastic tissue.



TEXT-FIGURE 2.—Effects of X ray and 5-FUDR on the uptake of ^{3}H-TDR *in vitro* into patient with multiple lesions from Hodkgin's disease.



TEXT-FIGURE 3.—Effects of diethylstilbestrol and X-ray castration on incorporation of ^{3}H-TDR *in vitro* into the DNA of human breast carcinoma.

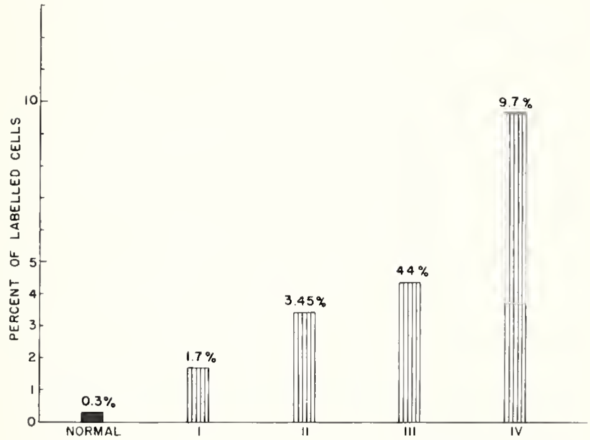
The *in vivo* incorporation of ^{3}H-TDR into DNA was also studied to determine the rates of cell proliferation in a variety of human tissues, including human tumors. The percent cells incorporating ^{3}H-TDR into DNA and the percent of labeled mitosis were studied as a function of time after a pulse label of thymidine. In some instances, double label was used: The individual was given an injection of ^{3}H-TDR and ^{14}C-thymidine incubated *in vitro*. With these techniques, the length of the cell cycle or the regeneration times for various human tumors were measured. Preliminary results are presented in table 4. An interesting observation from these data was that the estimated cell cycle time of alveolar cells from mammary gland in postmenopausal women is approximately 2–2.5 months. Estradiol therapy reduces this generation time to approximately 8.5 days. The portion of the alveolar cell cycle most markedly affected is the DNA synthesis period.

Another observation based on the above technique is that human tumors do not necessarily grow or regenerate faster than normal tissue. In many instances, the length of the cell cycle is longer than that of normal tissues of the same origin. There-

TABLE 4.—Estimated length of cell cycle *in vivo*

Human tissue	Estimated generation time
Epidermis	1–1.5 mo.
Intestinal epithelium	17–22 hr.
Polychromatophil normoblasts	15–18 hr.
Alveolar cells, mammary gland:	
Postmenopausal	2–2.5 mo.*
Stimulated by estradiol	8.7 days*
Hodgkin's disease	1.5 days
Adenocarcinoma of the breast	4.5 days
Adenocarcinoma of the breast	8.5 days
Carcinoma of the breast	3 mo.
Adenocarcinoma of the bowel	24 hr.
Squamous cell carcinoma	2.5–3.0 days
Melanoma	8.5 days
Carcinoma of the colon	25–30 hr.
Carcinoma of the liver	10 days

* Data on normal breast are incomplete because of the limited number of patients and the high variability due to hormonal differences in each individual at time of biopsies.



TEXT-FIGURE 4.—Relation between the histologic grade (1–4) of malignancy of urinary bladder carcinoma and nuclear uptake of ^{3}H-TDR.

fore, the rapid increase in mass of a tumor may not necessarily be related to increased proliferation but to a different type of division resulting in survival of daughter cells. On this assumption, it can be understood why chemotherapeutic agents that have been designed to inhibit active cellular metabolism show such a high degree of toxicity with such a low regression rate in human tumors.

In conclusion, despite the many pitfalls which can influence the interpretations of results, any procedure which permits the patient to be his own chemotherapy testing system should be superior to any known animal or *in vitro* assay. In the method described above, there appears to be an 80% chance of determining at the end of 7 days of therapy whether a cancer patient will respond to therapy. Since the clinical response rate is approximately 20%, this procedure offers the clinician an 80% chance of rapidly determining the 1 of 5 individuals who will respond to therapy. The method may also be useful with patients who do not show a significant metabolic response after 1 week of therapy or with patients who show a marked stimulation of DNA synthesis; it will allow the physician to withdraw or change therapy early in treatment. So few patients respond favorably to chemotherapy that this approach to chemotherapy testing may spare many patients the severe side effects of prolonged drug toxicity. Hopefully, further experience with the DNA testing system will justify its routine use in palliative chemotherapy.

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Enzymic Synthesis of Steroid Sulfate by Mammary Cancer and Its Clinical Implications[1, 2]

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CONJUGATION of steroids with sulfuric and glucuronic acids has, for a long time, been considered to be a catabolic reaction confined to the liver. Only recently have other tissues been shown to contain enzymes for synthesizing conjugates. Both hormone-producing tissues, such as the adrenal, placenta, ovary, and testis, and tissues which do not secrete hormones, such as intestine and prostate, have been shown to possess conjugate-forming ability. The significance of conjugate formation by these tissues is not entirely clear. Apparently, in many instances, conjugation indeed represents a major process in the metabolic inactivation of steroid hormones, but in other instances, it seems concurrent with their metabolic transformations *(1)*.

Our interest in studying steroid conjugation systems in mammary cancer originated from our clinical observation that, in metastatic cancer of the breast, a relationship exists between the site of metastases and their responsiveness to hormonal therapy *(2)*. The most striking observation was that liver metastases often are refractory to hormonal treatment, whereas metastatic lesions elsewhere are more hormone responsive. It was thought that liver, as a major site for steroid conjugation, may be an important factor influencing the responsiveness to hormonal alterations. There was a complete lack of knowledge whether metastatic involvement of liver might increase or reduce the ability of liver to synthesize steroid conjugates. One of our major objectives, then, was to compare the steroid-conjugating systems of various metastatic lesions in different sites and also with those in the primary tumors.

METHODS

Tumor tissue removed for biochemical studies was either primary breast cancer, excised at the time of mastectomy, or soft-tissue metastases or primary inoperable breast cancer, removed at the time of adrenalectomy. Most of the patients undergoing adrenalectomy also had, in other sites, demonstrable metastases in active progression. At the time of adrenalectomy, the abdominal cavity was explored routinely, and both normal and metastatic tissues from the liver and/or other sites were also obtained for biochemical studies.

All tissues obtained fresh from surgery were worked up within 15 minutes. The methods for the preparation of tissue, incubation, extraction, and final estimation of steroid sulfate synthesized have been detailed in previous publications by us *(3, 4)*. Briefly, the minced tissue was homogenized in sucrose and centrifuged. Portions of the supernatant ($12{,}000 \times g$) containing enzyme were then incubated with 100 μmoles of pH 7.6 Tris buffer, 5 μmoles of adenosine triphosphate (ATP), 2 μmoles of $MgCl_2$, 0.1 μmole of K_2S0_4, approximately 5 μc (0.10 μmole) of $H_2{}^{35}S0_4$, and 0.05 μmole of steroid in a final volume of 1.0 ml. The steroids routinely used were dehydroepiandrosterone (DHEA), pregnenolone, deoxycorticosterone (DOC), estradiol-17β (E-17β), estrone, estriol, and testosterone. Incubation was for 1 hour at 37°C.

The reaction was stopped by chilling, and 1 ml of 2 N NH_4OH, saturated with $(NH_4)_2SO_4$, was added to the incubation mixture, which was then extracted 3 times with 3 ml of ethyl acetate. The ethyl acetate extract was brought up to 10 ml and was backwashed with seven-eighths saturated $(NH_4)_2SO_4$. Portions were counted in a scintillation counter with 10 ml of a scintillation fluid. Steroid sulfate synthesized by all tissues was calculated in picomoles (10^{-12} moles)/mg of protein/hour. Total protein content of the tissue was estimated by the biuret reaction. The biochemistry of the enzyme, characterization of the steroid sulfate synthesized, and the reproducibility of the assay method were detailed in an earlier paper *(3)*.

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[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

RESULTS

Enzymatic Sulfation of Steroids by Neoplastic Tissues

The presence of enzyme systems to form steroid sulfate esters in the mammary gland and mammary cancer tissues was demonstrated independently by investigators from two laboratories in two continents *(3, 5)*. These studies are of considerable significance, for they have questioned whether these chemical reactions are concerned with metabolic transformation of steroid hormones by mammary cancer tissues.

Work from our laboratory disclosed the following: 1) Mammary cancer tissue contains two populations of cells—one possessing enzyme activity and one lacking the enzymes capable of forming sulfate esters with a variety of steroid hormones; 2) sulfurylation of different steroid hormones by normal liver tissue may vary from one individual to another, but, as the relative activity within a single individual is very close to being constant, DHEA is the most efficiently sulfurylated, and the ratio of each of the others to DHEA is about the same; 3) the pattern of sulfurylation of steroid hormones in mammary cancer tissue, however, is not the same as that in normal liver tissue, especially with respect to E-17β. Some mammary cancers can conjugate estradiol much more efficiently than DHEA, but the case with others is reverse.

An interesting and perhaps very important finding is the observation that the ability to form steroid sulfate by the metastatic lesion does not seem necessary to follow the pattern of either the normal counterpart or the primary mammary cancer tissue. In our earlier study, we found that there were distinct differences between the primary tumor and the liver metastases *(3)*. In one instance, the primary breast tumor had no detectable activity, but the liver metastases were highly active. Thus the primary and metastatic mammary cancers may differ in their ability to conjugate steroid hormones. We have been particularly interested in studying the synthesis of steroid sulfate by normal liver and metastatic liver tissues in patients with disseminated breast cancer. In 18 patients, both normal and metastatic liver tissues were removed at the time of adrenalectomy. They were studied for their capacity to form steroid sulfate. In most patients *(12)*, the ability of the liver to synthesize steroid conjugates was greatly impaired when normal liver tissue was replaced by neoplastic tissue. In the remaining 6 patients, however, steroid sulfate synthesis was considerably enhanced in the neoplastic liver tissue. When the response to adrenalectomy was assessed in these 18 patients, the ratio of DHEA sulfate to estradiol sulfate in 3 patients was greater in the metastatic liver tissue than in the normal liver, but in 1 patient the ratio was lower (table 1). In contrast, in 14 adrenalectomy failures, the ratios of DHEA sulfate and estradiol sulfate in the metastatic liver tissues were either less than or equal to those of their normal counterparts. Eight of the nonresponders had a decreased ratio of DHEA sulfate to estradiol sulfate in their neoplastic liver tissues, and the remaining 6 had almost identical ratios in both the normal and neoplastic liver tissues. Also of interest is that 5 of these 18 patients had metastatic liver tumors that synthesized more steroid conjugates than did their normal liver tissues. These observations led to the following conclusions: 1) In most cases, the capacity to sulfurylate steroid hormones is markedly lower in liver tissue involved with metastasis than in the corresponding normal tissue. Thus neoplastic liver cannot metabolically inactivate steroid hormones as effectively. 2) In some cases, there is considerably more enzyme activity for the synthesis of steroid sulfate in the neoplastic liver tissues than in their corresponding normal liver tissues. In these cases, the neoplastic livers seem to retain the physiological activity of the normal tissues.

Sulfation of Steroid Hormones by Breast Cancer Tissue and Clinical Correlation to Response to Adrenalectomy

We have already correlated clinical results of adrenalectomy with steroid sulfotransferase assay in tumors *(6)*. In the present paper, the results of our enlarged series will be given. Mammary cancer tissue was obtained from 79 women with disseminated breast cancer who were scheduled to undergo bilateral adrenalectomy. The patients were all postmenopausal. Menopause was either spontaneous or induced by surgical castration. The age ranged from 36–76 years, with a median of 55 years and a mean of 54.5 years.

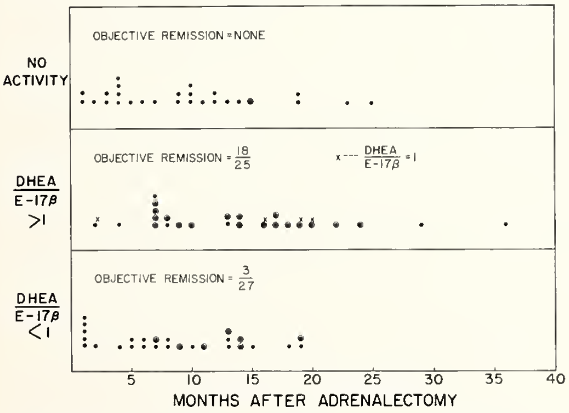
Of these 79 patients, 27 patients had mammary cancer tissues negative for enzyme activity. These patients did not respond to adrenalectomy. The

TABLE 1.—Synthesis of steroid sulfate by normal and metastatic liver tissues in 4 adrenalectomy responders

Patients	Age (years)	Tissue studied	Steroid sulfate synthesized ($\mu\mu$moles/mg protein/hour)				
			DHEA	Pregnenolone	E-17β	Testosterone	Ratio*
L.T.	54	Normal liver	268.2	270.1	88.2	173.9	3.0
		Liver metastases	163.3	70.3	91.1	49.5	1.7
M.B.	65	Normal liver	1150.0	1082.0	709.0	738.0	1.6
		Liver metastases	1065.0	496.0	469.0	291.0	2.2
B.M.	46	Normal liver	40.5	40.9	24.2	5.5	1.6
		Liver metastases	13.7	10.5	5.5	2.0	2.4
N.K.	57	Normal liver	46.5	30.5	24.5	24.0	1.8
		Liver metastases	87.0	70.0	39.0	28.1	2.2

* DHEA : E-17β.

ratio of DHEA sulfate to estradiol sulfate was greater than unity in 21 tumors, equal to unity in 4, and less than unity in 27. Table 2 shows clearly that breast cancer containing enzymes that sulfurylated DHEA more efficiently than E-17β regressed after adrenalectomy. In contrast, tumors that sulfurylated E-17β more efficiently than DHEA rarely responded to the ablation of the adrenal glands. Perhaps the most important finding in this study was the observation that tumors lacking demonstrable steroid sulfate synthesis uniformly failed to respond to adrenalectomy. It is even more striking when the survivals of these patients were evaluated. The data in text-figure 1 show that patients with tumors forming more DHEA sulfate than estradiol sulfate survived significantly longer. Furthermore, of the 25 patients whose tumors had ratios of DHEA sulfate to estradiol sulfate greater than unity, 18 or 72% were still alive. In contrast, only 8 or 12% of the 54 patients whose tumors either lacked the enzyme activity or sulfurylated estradiol more efficiently than DHEA were alive. These data strongly suggest a correlation between the laboratory results of this *in vitro* study and the clinical results of adrenalectomy.



TEXT-FIGURE 1.—Correlation between steroid sulfotransferase activity in tumor and survival after adrenalectomy. ⊙ = living; ● = dead.

TABLE 2.—Conjugation of steroid hormones and response to adrenalectomy

Number of Patients	Sulfokinase activity in tumor DHEA:E-17β ratio	Response to adrenalectomy	
		Remission	Failure
27	None	—	27
21	>1	17 (81%)	4
4	=1	1	3
27	<1	3 (11%)	24
Total 79		21	58

Predictive Value of Measurement of Steroid Sulfate Synthesis by Primary Breast Cancer

Our investigation so far has produced convincing data that assay of enzymes for synthesis of steroid conjugates by tumor tissue may be used for selection of adrenalectomy. The practical value of this assay for selection of patients for adrenalectomy, however, is yet to be determined, since more than half of all patients undergoing adrenalectomy have metastatic tumors in organs, such as lungs, bones, and central nervous system, that cannot routinely be removed for assay. An important question to be answered is how often primary breast tumors differ from the recurrent tumors in their ability to synthesize steroid conjugates. In other words, can measurement of steroid sulfate synthesis by the primary breast cancer at the time of mastectomy predict the future response to endocrine ablative procedures? Further, does assay of steroid conjugate synthesis in the primary tumor have any prognostic value for patients undergoing radical mastectomy?

Primary mammary cancers removed at the time of mastectomy were studied in 92 patients; 56 of these patients had radical mastectomy and 36 simple mastectomy. Simple mastectomy was done because of the age of the patients, medical contraindication, and in a few instances because of locally advanced breast cancers without distant metastases. Table 3 shows that, of the 9 patients having recurrence, they were almost equally divided among groups whose tumors had either no measurable sulfotransferase activity or had ratios of DHEA sulfate and estradiol sulfate of <1. In contrast, none of the 10 patients whose tumors had greater activity in forming DHEA sulfate than estradiol sulfate (ratio > 1) had evidence of metastases. In the simple-mastectomy group, similar results were observed. The data provide some evidence that the incidence of recurrence and early metastases after mastectomy is much higher in patients whose tumors have no enzyme activity for steroid conjugate synthesis or whose tumors have much greater enzyme activity in synthesizing estradiol sulfate than DHEA sulfate. However, these data are only preliminary, since most patients in this series had their mastectomy less than 2 years ago.

TABLE 3.—Assay of steroid sulfate in primary breast cancer and clinical correlations with incidence of recurrence

Sulfokinase activity in tumor DHEA:E-17β ratio	Radical mastectomy		Simple mastectomy	
	Number without recurrence/ total No. patients	Number with recurrence/ total No. patients	Number without recurrence/ total No. patients	Number with recurrence/ total No. patients
None	17/21	4/21	4/9	5/9
>1	10/10	0/10	4/5	1/5
>0.6	7/8	1/8	4/6	2/6
<0.6	13/17	4/17	6/16	10/16
Total	47/56	9/56	18/36	18/36

We also wished to determine whether primary tumor and recurrent tumor differ greatly in their ability to form steroid sulfate. Of the 27 patients who developed metastases, only 9 had soft-tissue lesions which could be removed for assay of their sulfotransferase activity. The data in table 4 reveal that tumors from only 2 patients differed significantly in their ability to synthesize steroid sulfate formation; in patient E.B. the ratio of DHEA sulfate and estradiol sulfate rose from less than unity in the primary tumor to greater than unity in the recurrent tumor, and in patient J.W. the primary tumor was negative in enzyme activity, whereas its metastatic lesion showed a ratio of >1. In the remaining 6 patients, the primary tumors and their metastatic lesions did not differ significantly in their ability to synthesize steroid sulfate. We would have been wrong if we assumed that patients E.B. and J.W. would not respond to adrenalectomy, since patient E.B. had a lack of sulfotransferase activity and patient J.W. had a ratio of DHEA sulfate to estradiol sulfate of <1 in the tumor. The assay of the recurrent tumors in patients E.B. and J.W. predicted that both patients would respond to adrenalectomy. In contrast, 7 patients with metastases underwent bilateral adrenalectomy without assay of steroid sulfotransferase activity, since the metastatic lesions were in the bones or visceral organs. However, the assay of the enzyme activity of the primary tumors predicted that none would respond to adrenalectomy, since 4 tumors were inactive and 3 had ratios of <1. Results in table 5 clearly show that all 7 patients failed to respond to subsequent adrenalectomy. If the data in tables 4 and 5 are combined, the results disclose that assay of sulfotransferase activity in the primary tumor is highly accurate in predicting subsequent response to adrenalectomy when metastases develop. However, no justifiable conclusion can be made based on the few patients studied.

Sulfate-Activating Enzymes in Neoplastic Tissues

The correlation between clinical and laboratory findings led to our further investigation of steroid sulfate formation in these tumors. Three enzymes are necessary to form steroid sulfates: sulfate adenylyltransferase, adenylylsulfate kinase, and one of two sufotransferases—3β-hydroxysteroid sulfotransferase or estrone sulfotransferase. These enzymes catalyze the following series of reactions:

$$\text{ATP} + \text{SO}_4 = \text{APS} + \text{PP}_i \text{ (pyrophosphate)}$$

$$\text{APS} + \text{ATP} = \text{PAPS (adenosine 3'-phosphate 5'sulphatophosphate)} + \text{ADP (adenosine diphosphate)}$$

$$\text{PAPS} + \text{steroid} = \text{steroid sulfate} + \text{PAP}$$

The first two enzymes are necessary to form "active

TABLE 4.—Comparison of steroid sulfate synthesis in the primary and recurrent tumors*

Patient	Primary tumor			Recurrent tumor			Response to adrenalectomy
	DHEA sulfate	Estradiol sulfate	Ratio	DHEA sulfate	Estradiol sulfate	Ratio	
A.B.	0	0	—	0	0	—	None
E.B.	7.5	9.4	0.8	19.5	13.0	1.5	Good
L.L.	9.4	19.7	0.4	168.3	174.5	0.9	Too early
F.De.	37.2	112.7	0.3	33.6	100.0	0.3	None
J.W.	0	0	—	6.7	6.4	1.0	Good
B.H.	1.1	1.3	0.8	54.4	110.0	0.4	None
L.B.	17.5	13.8	1.2	184.5	81.5	2.2	No change
D.R.	0	0	—	0	0	—	None

* Primary tumors were removed at the time of radical or simple mastectomy, and recurrent tumors at the time of adrenalectomy.

TABLE 5. Assay of steroid sulfate in the primary breast cancer and clinical correlations with subsequent response to adrenalectomy

Patients	DHEA sulfate:estradiol sulfate at time of mastectomy	Response to adrenalectomy
A.C.	0	None
P.P.	0.5	None
E.G.	0	None
C.K.	0.4	None
B.C.	0	None
D.R.	0	None
M.C.	0.6	None

sulfate," PAPS. Lack of any one of these enzymes results in the failure of steroid sulfate formation by the tumor preparations. Purification of sulfating enzymes from yeast enabled us to determine whether the inactive tumor preparation lacked the sulfate-activating enzymes or the sulfotransferases *(6)*. Our studies with preparations from human breast tumors which were inactive in the formation of steroid sulfates revealed that these preparations lacked the 2 sulfate-activating enzymes, sulfate adenylyltransferase and adenylylsulfate kinase, and had measurable amounts of 3β-hydroxysteroid sulfotransferase and estrone sulfotransferase. We also observed that the ratio of DHEA sulfate to estradiol sulfate was unaffected by the addition of an excess of either sulfate adenylyltransferase or adenylylsulfate kinase, or both. Thus the ratio might have been controlled by the relative amounts of the 2 sulfotransferases, rather than by an effect of steroids on the sulfate-activating enzymes *(6)*.

These observations led us to consider another area of research: the relationship between mucopolysaccharide metabolism and sulfate metabolism in these tumors. Human breast cancers have never been investigated in this regard. The possibility that some human breast tumors do not synthesize sulfated polysaccharides because they lack the sulfate-activating enzymes is interesting. Since acid mucopolysaccharide (AMPS), though not all AMPS in one tissue, are felt to be ubiquitous, the question arose: Could there be an impairment in the synthesis of these substances in some breast tumors, which may or may not be related to the other property of these tumors, *i.e.,* the lack of response to adrenalectomy?

It, therefore, appears that the sulfation of steroid hormones by the mammary cancer may be related to at least two distinctly different biochemical and physiological aspects—mucopolysaccharide metabolism and steroid hormone transformation. Although it is unclear how sulfation of steroid hormones by mammary cancer may be related to hormone responsiveness of the tumor, the possible role of sulfation in the biochemical transformation of hormonal precursors to the physiologically active hormones may be of considerable importance in our understanding of the pathogenesis of mammary cancer.

SUMMARY

Conjugation of steroid hormones by mammary cancer tissue was studied in 92 patients undergoing mastectomy for primary disease and in 79 patients undergoing bilateral adrenalectomy for their metastatic disease. An assay of the steroid-conjugating enzyme system was performed in all primary tumors, recurrent soft-tissue lesions, and all metastatic lesions that could be removed at the time of operation. Primary tumors from 30 of 92 mastectomy patients were inactive; the ratios of DHEA sulfate to estradiol sulfate were > 1 in 15 patients and < 1 in 47 patients. Only 1 of the 15 patients with a greater-than-unity ratio in the tumor had evidence of metastases, whereas 17 of 47 patients with tumors having a < 1 ratio and 9 of 30 patients

with inactive tumors had metastases. The limited data suggest that assay of steroid sulfotransferase activity in the primary tumor has a prognostic value. In the 79 adrenalectomy patients, 27 patients had inactive tumors, and all failed to respond to adrenalectomy. Seventeen of twenty-one recurrent breast cancers containing enzymes that sulfurylated DHEA more efficiently that E-17β (*i.e.*, a ratio of DHEA to E-17β of > 1) regressed after adrenalectomy. In contrast, only 3 of 27 tumors that sulfurylated E-17β more efficiently than DHEA regressed after adrenalectomy. Therefore, assay of steroid sulfotransferase activity in the tumor tissue is a valuable test for predicting response to adrenalectomy.

EPILOGUE

Predictions, Predictions, Predictions,
We hear them loud and clear
Scientists' claims delight the clinicians,
Like a man lost in the desert, looking for water
Elated, crying out, water on the horizon!
Not quite, it was a mirage.
Do not despair, real predictions on the way,
When this happens, to Stockholm we go!

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DISCUSSION

Dr. Hall: Dr. Heidelberger, there were 3 different doses on your slide: 0.3 mg/kg (because that was given several times a day?), 27 mg/kg, and 2.5 mg/kg. Thus I could not ascertain what you believe a pharmacologically feasible dose is.

Dr. Heidelberger: It took Dr. Ansfield quite a while to establish a dosage, and this is a real phase-I study. Originally, he found that 27 mg/kg/day, 1 shot fast, was tolerated for about 5 days. But he did not see any clinical responses. So he increased the duration of the course and cut down on the dosage. The schedule that seems to be producing responses is 0.3 mg/kg given every 4 hours throughout the day, to a total of about 2.5 mg/kg/day. There have also been some studies in which this was used as a loading dose, and then a maintenance shot of 2.5 mg/kg was given once, twice, or three times a week. We are doing our prospective correlation at a dose of 0.3 mg/kg given every 4 hours. In one of the slides, the blood level was higher after the higher dose and was maintained a little longer. At face value, this appears to show that the treatment should be continued for at least 10 days to produce a response. We are hoping to predict the percentage of unchanged trifluorothymidine excreted. This percentage appears to be slightly dose dependent.

Dr. Mihich: Dr. Heidelberger, you mentioned that trifluorothymidine is toxic to bone marrow but not to the intestinal mucosa. Is this differential tissue toxicity correlated with either different uptake or with decrease in metabolism or in anabolism?

Dr. Heidelberger: This we did only once in the mouse. We did not see very much. Dr. Rogers is studying this point now in dogs. In man, we have unfortunately not had enough radioactive material to give to a patient before surgery.

Dr. Braunsberg: Could I ask Dr. Dao some questions? The first one concerns your earlier work with liver metastases in which you related the differences between normal liver tissue and metastatic liver tissue. These ratios involve two measurements; yet the differences are not very great. Do you really think that this is significant? Do you know the variability of these ratios as you do repeated assays, for instance? And, when you get such small differences, are they in fact significant? The second question is really on the same line. Is it not most important to assess from the behavior of the primary tumor what you are going to do with this patient later on, because, when the patient comes to the stage where he may need adrenalectomy, you must know from your data on the primary tumor whether adrenalectomy should be done? The primary tumors are notoriously heterogeneous. If you sample a large tumor at different areas in a large mass, how variable are these ratios? Bearing in mind that the ratio itself must vary because of the measurement of variability—and superimposed on that is the heterogeneity of the tissue—with what accuracy can you measure this?

Dr. Dao: To answer your first question, which certainly is a valid one, if you are familiar with the method we use, there is no question that there is a very significant difference here. First of all, we do duplicates in each hormone. There is absolutely no overlapping, and the duplicates always are almost identical. I want to make a point of the reason we use dehydroepiandrosterone (DHA) and estradiol. If you look at the synthetic enzymes of different steroids or sulfate formation in the tumor or liver, the DHA is constant with respect to other steroids. The one exception is estradiol. This is why we use this ratio. The point is very valid that, if you are dealing with a ratio close to unity, it is questionable if 0.8 or 0.9 is meaningful. We have to have a large series on hand to study.

In reply to your second question about the heterogeneity of the tumor, it is curious that so far we sometimes emphasize too much the heterogeneity of the tissues. We have tested the influence of the heterogeneity of the tumor. We have taken tumors from different areas several times and also from different areas in the primary tumor; we have found no striking difference in the enzyme contents.

Participant: Is sulfokinase a microsomal enzyme or is it a soluble enzyme?

Dr. Dao: It is soluble.

Dr. Hilf: Perhaps something that we consider too much is the question of heterogeneity. We need to find out just exactly how much variation or heterogeneity we may get. In the series that we studied, we tried to develop techniques in which we could use as small a piece of tissue as possible so that we could make many assays for any one given lesion. We found that the variability was certainly well within reason; ranging from 5–10%, it was in the same order that we found in tumors from animals with transplantable tumors that we have maintained for years. So it is not really as bad as one might have anticipated.

Dr. Tannock: No matter how small your sample size is, I am sure it is large compared with the sort of distances between blood vessels and regions of necrosis within a tumor, which is about 100 μ. So you are not really sampling cells in different environments which is important. When Dr. Sky-Peck worked in the same laboratory that I worked in, in England, we did some studies on thymidine uptake into tissue slices. We found that we could get very good correlation with what we would measure *in vivo* if we incubated the tissue slices in high-pressure oxygen. But, if we did not do that, we would get a rim of labeled cells around the outside but less uptake or no uptake of thymidine in the center. Without incubation in high-pressure oxygen, obviously the counts are going to be in error. Also, if you use this technique during therapy, you are very safe if you just approach this empirically, as I think most of you have done, by saying the change of labeling index may be associated with a given type of response. If you try to analyze in terms of cell-cycle parameters, you may get into difficulties because many cells that you would be studying are dying because of the therapy you are applying.

Dr. Sky-Peck: What you say is true. Our systems are O_2-CO_2 systems, and they are rather thin sections, so we do not have a diffusion problem. We compared *in vivo* labeling with *in vitro* labeling and found that both labeling conditions are fairly much the same. As a matter of fact, I had many slides that showed *in vivo* tagging of individual cells. Heavy labeling could be seen around the periphery of tumors but none in the center. You do have the circulation problem, the same as you do the diffusion problem.

In addition, I did not imply that I was trying to study kinetics under abnormal conditions. I realize that you run into this problem with populations. One thing we did do —I wish somebody could get some answers to this—was to try to study cell kinetics under normal conditions and then to repeat the whole study on cell kinetics under therapeutic conditions to see if one could actually alter the cycle in any way, shape, or form. Our work with radiation indicated that, when cells regrew, they did so at exactly the same rate as they did before they were irradiated. But we have no information on chemical changes. This, of course, takes into consideration the difference in kinetics from small samples to larger samples also.

Dr. Skipper: Dr. Heidelberger, is RNA or protein synthesis significantly inhibited at high concentrations?

Dr. Heidelberger: We have not found inhibition in experiments with Ehrlich and L1210 cells in animals. Our tissue culture experiments lead us to believe that high concentrations probably would not inhibit synthesis.

Dr. Skipper: I asked that question because you mentioned this and then Dr. Sartorelli mentioned the effects on nucleotide reductase. We are seeing so much now at the biochemical level. It seems that it is carrying over to timing of therapeutic schedules, which deal again with those kinetics about which we were talking. Every example that I know—and there must be 5 or 6 now—of effects at a biochemical level seems to be DNA specific and is not blocking RNA nor protein to any degree. Drug spacing becomes very important. This does not always carry over to agents that block RNA or protein synthesis.

Dr. Sartorelli: In our case, I did not want to imply that this is the sole biochemical effect. This is the major biochemical effect. You achieve this at concentrations that do not do other things to cells. With therapeutic doses, you can get other effects on RNA and protein synthesis. In fact, in less sophisticated systems, like *Escherichia coli*, the primary lesions seem to be inhibition of RNA synthesis.

Dr. Heidelberger: Dr. LePage and Dr. Sartorelli, there is some controversy about the mechanism of action of cytosine arabinoside (Ara-c). Dr. Fischer is a reductase inhibitor advocate and Dr. Seymour Cohen is a DNA polymerase inhibitor advocate. Dr. LePage showed very interestingly that arabinosyl-6-mercaptopurine also inhibits reductase, and Dr. Sartorelli showed that this semicarbazone also inhibits the reductase. Dr. LePage's other compound, arabinosyladenine (Ara-a), inhibits DNA polymerase. What is the cross resistance, if any, between these compounds in an Ara-c-resistant line? This might help to pinpoint the importance of the two Ara-c pathways.

Dr. LePage: I am afraid the cases you cited are not much help because Ara-a, in the form of the triphosphate, is also an inhibitor of the reductase and the polymerase, although the effects of Ara-a on the polymerase are perhaps more profound in that they occur at a lower concentration.

Dr. Sartorelli: Dr. Fischer tried one of the other derivatives in a line which was resistant to Ara-c, and I think this line was still sensitive but it had some decrease in sensitivity. If we take the hydroxy and move it over to the 3 position, we still have a very active antitumor agent with a spectrum of activity greater than that of the 5-hydroxy derivative. Both of these compounds are inhibitors of the ribonucleotide reductase and seem to owe their action predominantly to that site of action. That clearly is not the whole story because there are slight differences in the kinetics of inhibition and the mechanism of enzyme inhibition, so that we can get a cell line which is completely resistant to this agent and is not resistant to the 3-hydroxy derivative. In the cell line, we can see the blockade of the enzyme *in vivo*, as measured by cytidine conversion to deoxyribonucleotides, but the duration of this block is extremely short and we cannot even tailor our therapy to block it.

Dr. Bertino: This trifluorothymidine looks like a very exciting agent because it does not have gastrointestinal toxicity. This is a tremendous advantage over 5-fluorouracil. In terms of metabolism and the different ratios between responding and not responding, are you also finding that the so-called "nonresponsives" are not getting toxic to the drug? Maybe what you are seeing is that you just are not giving enough drug; if so, perhaps another way of giving the drug will increase your responses. Do you have any data on whether or not those patients who metabolize the drug also do not have bone marrow toxicity?

Dr. Wolberg: We tried analyzing the degree of various parameters, as far as correlating response to the amount of drug given, toxicity, bone marrow, or whatever. We could find no correlation.

Dr. Prager: Dr. LePage, when you refer to the cytidylate reductase, does this imply greater specificity for a type of enzyme or is this merely the substrate that you selected? My next question relates to the phosphorylation of your 6-mercaptopurine arabinoside. You mentioned that the nucleotide was not as good an inhibitor of tumor growth as the nucleoside. Does the nucleotide actually get into the cells?

Dr. LePage: To answer your first question, there does seem to be specificity, although it is hard to be sure of this because the enzyme is very complex. Dr. Sartorelli indicates that this is one enzyme, on the basis of studies of *E. coli* and embryos. It may or may not be true in mammals; but, if we suppose that it is true, our own results show that, when you use cytidylate as the substrate you get inhibition and when you use guanylate or adenylate as the substrate you do not get inhibition. Thus you can say there is a certain specificity. This is an enzyme that involves a series of activators and inhibitors. The reduction of cytidylate involves activation of thymidine triphosphate, as I recall, and is inhibited by deoxyadenosine triphosphate and deoxyguanylate. Thus it is complex enough so that there may be other explanations and specificity for the cytidylate substrate, but that is the way it looks at the moment. And that is the way the *in vivo* data point because, if you use a variety of precursors for labeling your DNA, the only one that is affected is the labeling of pyrimidines and the effect is primarily on the cytidylate in the

DNA and to a lesser extent on the thymidylate of DNA. This could be indirect because some of the thymidylate probably comes from cytidylate via the deaminase. The nucleotide has less activity and, since there is phosphomonoesterase in the preparations, we cannot help but conclude that the decreased activity seen results from formation of nucleoside from nucleotide in the same extracts. These are done in cell-free extracts, so that penetration of the nucleotide is not involved.

Dr. Sartorelli: In our system, we can inhibit the reduction of all of the nucleotide diphosphates, the deoxy counterparts, and the concentration required for 50% inhibition of the reaction is approximately the same. Dr. LePage, in view of the complexity of the system, could any of the reactants in your preincubation studies prevent the inactivation?

Dr. LePage: We have not seen any sparing effects. Relative to what Dr. Skipper was saying—if this agent is indeed effective—I find only one reaction, the reductase reaction. This may be of great advantage because we have an agent that inhibits DNA synthesis only in S phase and does not prevent cells from coming around the cycle to the S phase. To have a simple situation like this, where you affect only S phase, is probably unique. Even hydroxyurea probably affects reactions other than DNA synthesis and prevents cells from coming into the S phase. It would be a distinct advantage to have the cells go on around and then one after another get into the S phase.

Dr. Zelen: Three years ago, Dr. Perry, Dr. Zimmerman, and I published a study on the total thymidine uptake for acute lymphoblastic leukemias. The groups studied were normal children, children who were completely free of the disease or maintained remission, children who were in complete remission with less than 5% blast cells, children who were in partial remission, and children who were in relapse. What we noticed was the more serious the disease the higher the uptake. But an interesting phenomenon was that the worse the disease the more variability in the uptake and the harder it would be to use it for any kind of a predictive system. We were interested in trying to predict in advance when someone in remission would go into relapse a few weeks before it would happen; we were unsuccessful.

Dr. Dao, did you correlate your ratios with the free periods or is there any correlation?

Dr. Dao: Yes, we did, but I do not think the correlation means anything because we had only a few people who had had a relapse after radical mastectomy. There is some correlation in that a long free period is better.

Dr. Bertino: We find the same thing: As you get higher and higher uptakes, your variability gets greater and greater. In some of our very active cases, it is very difficult to get a statistical difference between 7-day periods. Usually these diseases are so active anyway that they do not respond to therapy.

Dr. Lemon: Dr. Sky-Peck, have you done any studies on patients after 7 days of diethylstilbestrol treatment? Sometimes the clinical remission, of course, comes weeks later.

Dr. Sky-Peck: I had 1 patient to whom we gave diethylstilbestrol. At the end of the 7-day period, there were marked increases in the percentages of cells labeled, the specific activity, and the so-called free thymidine state by dilution techniques. When we got to the 21-day point, we got into a so-called regression, and the percentage of cells labeled and the specific activity dropped; yet the so-called pool state remained the same. We have seen this to occur a few times.

Dr. Lemon: It seems to be a most interesting observation because it would suggest a secondary effect on pituitary hormone excretion.

Dr. Ellison: We did studies similar to those described by Dr. Zelen. We used tritiated thymidine and incubation for one-half hour with peripheral blood of adults with acute myelocytic leukemia. At that time, we had very few complete remissions, and we found great variability. We could not link this variability to the activity of the disease, as exemplified by the number of blast cells in the marrow. The peripheral blood of normal volunteers and that of patients with solid tumors did not differ significantly. There was a very great variability in the same patients when the tests were repeated within several days.

PANEL VII

IN VIVO HISTOLOGIC STUDIES

Pathologic Features of Prognostic Import in Human Cancer[1, 2, 3]

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I WILL cover briefly some of the particular pathologic characteristics of tumors we have been studying as possible aids in prognosis. I will emphasize the value of determining the presence or absence of blood vessel invasion at the primary site in certain types of tumor, and will discuss in some detail the prognostic value of nuclear grade in cases of breast cancer.

DEVELOPING CRITERIA

First, however, I would like to comment about the general process through which such morphologic prognostic criteria are developed. This process might well be divided into "Phase I," "Phase II," and "Phase III" studies.

In Phase I, the pathologist attempts to demonstrate a significant relationship between one or more pathologic features and patient survival. Usually he does this retrospectively, by reviewing surgical and/or autopsy material from his own files and then relating the gross and microscopic findings to both the treatment and the outcome in each case. If a meaningful relationship appears between the morphology and the behavior of a given type of tumor, the pathologist sets up what might be called a Phase II study.

In Phase II, the point at issue is whether the pathologist can repeat his predictive results when reviewing his own histologic material as a set of "unknowns" without information about the subsequent clinical course.

If his results are good, then the investigation would move into Phase III, the point at which *other* pathologists are asked to use the given histologic criteria to predict clinical outcome from tissue sections reviewed as unknowns. If the proponent has succeeded in defining meaningful histologic criteria insofar as prognosis is concerned, others can repeat his results and another predictive pathologic feature will have been developed. At this point, however, many apparently worthwhile tests for assessing prognosis fail. Thus, although the original pathologist, using one or more particular histologic parameters he selected, may be able to predict with a high degree of accuracy the clinical outcome of patients with a particular type of malignancy, other equally well-motivated pathologists may be unable to reproduce his findings.

The most likely explanation of this failure is that, despite his analytic efforts, the proponent has not made himself aware of all of the factors that have gone into his own assessment. Consequently, he will not have communicated to the other pathologists *all* the criteria which they needed to make predictions as accurate as his. Sometimes this failure in communication can be resolved by intensive joint study, but at other times this impasse is never surmounted. If the proponent of a given "determinant" cannot successfully move his proposition through Phase III studies, despite the satisfactory completion of both Phase I and II studies a certain amount of confusion and ill will may appear as rather unfortunate by-products.

The inability of one or more groups of pathologists to reproduce the results of the proponent of a given predictive "test" simply means that, from a practical standpoint, the "test" has significance only in the hands of the original proponent. It does not mean that the original concept was erroneous. However, after appropriate Phase III studies have been completed by other pathologists without demonstrating the usefulness of the "test," the problem of further defining the criteria originally used by the proponent should revert to him. That is, if he still believes there is a wider application than in his own laboratory, he must provide evidence to this effect.

To facilitate this discussion of prognostic pathologic features, I have listed them in three general groups: 1) those related to the appearance of the cancer cells themselves, without regard to the sup-

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[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

[3] Supported in part by Public Health Service contract PH-43-68-704 with the National Cancer Institute.

porting host tissue; 2) those related to the apparent relationship or interaction of cancer cells and host tissue reaction at either primary or metastatic sites; and 3) those related to the apparent response of a given host tissue or organ to the presence of tumor at some other site in the body. In a sense, group 1 represents the growth potential of the tumor itself, group 2 represents the host response to the presence of tumor at the site in question, and group 3 represents the response of some distant tissue of the host to a systemic stimulus elaborated or provoked by the tumor.

In my outline below, I have considered tumor type and grade to be functions of intracellular and intercellular characteristics. In some organs, the type of carcinoma appears to have prognostic significance, and for a given tumor type the grade, or degree of differentiation, also appears to have predictive value. Tumor type is generally assigned on the basis of cellular features of the tumor without regard to the stroma; *i.e.*, identification is based on the presence of intracellular cytoplasmic products or the presence of such cytoplasmic products elaborated into the interstitial tissue. For example, the presence of keratin or mucin serves to identify squamous cell carcinomas or adenocarcinomas, respectively.

HISTOLOGIC BASES FOR PROGNOSIS IN CANCER

I. Appearance of cancer cells (includes tumor type and grade)
  A. Intercellular relationships
  B. Intracellular characteristics
    1. Nucleus (reproductive capability)
      a. Nuclear grade
      b. Number and type of mitoses
    2. Cytoplasm (differentiation)
      a. Amount
      b. Metabolic products or specialization
II. Direct cancer cell-host tissue interaction
  A. *In situ* cancer
  B. Invasive cancer
    1. Extent or volume of tumor at primary site, including depth of penetration of wall of viscus ("subgross" assessment)
    2. Patterns of growth and spread
      a. Local
        (1) Expansile, *e.g.*, "broad front," "bulky outgrowth," "pushing type"
        (2) Infiltrating
          (a) Perineural spaces
          (b) Perivascular spaces
          (c) Intravascular spaces (lymphatic or blood)
      b. Metastatic
        (1) Lymphatic
        (2) Blood vascular
        (3) Other
    3. Presence of "basement membrane" around invasive cancer cell aggregates
    4. Inflammatory cell response at primary or metastatic sites
III. Indirect cancer cell-host tissue interaction
  A. Bone marrow response
    1. Cellular production
      a. Granulocytes
      b. Erythrocytes
      c. Plasma cells
    2. Cellular destruction
  B. Lymph node, *e.g.*, sinus histiocytosis, and splenic response
  C. Endocrine organ response to tumor, or to related pathogenetic factors, *e.g.*, ovarian cortical stromal hyperplasia
  D. Malignancy-associated changes
  E. Hyperplasia, or increased turnover, of parenchymal cells in nonlymphoid organs, *e.g.*, liver

The bases for grading tumors vary somewhat more than those for "naming" tumors and, at times, include either cytoplasmic or nuclear features, or some of each. Thus, in some tumor-grading systems, the degree of cytoplasmic differentiation, *e.g.*, the extent of keratinization, might be combined with the mitotic index and the appearance of the nuclei to arrive at a tumor grade. Despite the problems of grading, however, within a given grading system, the grades for tumors of a particular organ may be of definite prognostic significance.

Nuclear grade is quite distinct from the general tumor grade to which we have referred. In the determination of the nuclear grade of tumor, only the nucleus is examined. Cytoplasmic features are irrelevant. At the same time, I believe it is erroneous to refer to nuclei as "differentiated," since "differentiation" is a cytoplasmic property, although perhaps one could refer to certain tumor cell nuclei as resembling those of mature normal cells.

NUCLEAR GRADE

For the past few years, we have been attempting to apply the nuclear grading system developed by Black and his colleagues to the prognosis of breast cancer *(1–3)*. We have done this work in conjunction with several pathologists including Dr. John Bell, Dr. Enrique Soto, and Dr. Andrew Tiltman.

We have endeavored to see whether the nuclear grade of breast cancers was correlated with patient survival and with the presence of various other histologic features, some of which were also thought to have predictive value. The spectrum of nuclear grade ranges from grade 3 with small, fairly uniform, regular nuclei containing dispersed chromatin, rare nucleoli, and infrequent mitoses, to grade 1 in which there are marked nuclear pleomorphism with many large nuclear forms, prominent nucleoli, marked irregularity of chromatin pattern, and numerous mitoses. All other things being equal, the carcinomas composed primarily or entirely of grade 3 nuclei have a better prognosis than those with grade 1 nuclei. The difference in prognosis is less clear between nuclear grade 1 versus nuclear grade 2 cases or between nuclear grade 2 and nuclear grade 3 cases.

Nuclear grade appears to be independent of, but is probably related to, other histologic features. Some of the interrelationships between pathologic features are shown in table 1, which is a modification of a table from an article by Bell, Friedell, and Goldenberg *(4)*.

Table 1.—Five-year survival among 127 patients with breast cancer

Nuclear grade (% surviving 5 years)	Negative BVI* Negative nodes	Positive BVI Negative nodes	Negative BVI Positive nodes	Positive BVI Positive nodes
1 (30)	8/12	1/4	3/15	0/9
2 (54)	11/12	1/3	7/20	8/22
3 (70)	11/12	3/4	6/11	1/3
Total	30/36	5/11	16/46	9/34
Percent	83	45	35	26

* BVI = blood vessel invasion.

CANCER CELL-HOST INTERACTION

In situ cancer was listed in the preceding outline under "Direct cancer cell-host tissue interaction," although one might perhaps have listed it equally as well under "Appearance of cancer cells," since by definition there is no stromal invasion. On the other hand, stroma must be present in the tissue section to make the diagnosis of carcinoma *in situ*, and there is no way of knowing if tumor-host interaction keeps the cancer cells from invading the stroma or if the tumor does not possess the capability to invade. I have placed *in situ* cancer in this category because I wish to stress the need for the presence of stromal tissue in the biopsy specimen before a diagnosis of carcinoma *in situ* can be made. Without stromal tissue, only a diagnosis of "malignant epithelium" is justified.

The pathologic diagnosis of carcinoma *in situ* implies that invasive cancer either is present elsewhere in the organ at the time the biopsy or surgical specimen is obtained, or can be expected to develop in that organ later. If invasive cancer is *not* found when the diagnosis of carcinoma *in situ* is made, this pathologic diagnosis has obvious prognostic implication and the patient must be followed carefully.

In some organs, it is possible to remove or destroy most of the epithelium in which the carcinoma *in situ* has developed or will develop. For example, cautery or cold-knife conization of the uterine cervix can effectively destroy carcinoma *in situ (5)*. In certain other organs, however, such as the larynx or urinary bladder, the problem is less easily solved. Removal or destruction of all of the epithelium in question is not feasible. The discovery of the "flat-type" of squamous carcinoma *in situ* of the bladder, as shown by Koss *et al. (6)* and Melamed *et al. (7)*, is an important predictor concerning the possible development of invasive and potentially lethal bladder cancer.

A few comments are in order on the items I have listed under the heading "Invasive cancer" in the outline. The extent of tumor at the primary site has been shown to be significant in the prognosis of many different tumor types, including carcinomas of the colon, breast, and cervix. Indeed, the clinical estimate of the extent of tumor in cases of cancer of the cervix, the clinical staging, has proved to be of much more prognostic significance than the histologic type of tumor seen in biopsy specimens. The volume of tumor at the primary site is also a factor to be considered when evaluating the prognostic significance of regional lymph node metastases. In our review of surgically treated cases of cervical cancer, we found that the predictive significance of positive regional lymph nodes was directly proportional to the volume of tumor at the primary site. Thus, in Stage I cases with regional node involvement, 50% survived more than 5 years. In Stage II patients with positive lymph nodes, only 15% survived more than 5 years. In patients with primary tumors so extensive as to require pelvic exenteration, only rare patients with positive nodes survived more than 5 years *(8)*.

Our studies of the spread of cancer of the cervix, particularly the review of giant histologic sections *(9)*, has convinced us that in the cervix, as elsewhere, the direct extension of tumor in perineural and perivascular spaces is a common pattern. For the most part these spaces, particularly the perineural ones, appear to be tissue spaces rather than endothelial-lined lymphatic channels, and the designation of tumor in these sites as being located in "perineural lymphatic spaces" appears incorrect. Instead, this pattern seems to represent the spread of cancer along pathways of focally diminished tissue resistance. Since nerves and vessels penetrate even the most dense tissues, it is reasonable to explain the presence of tumor in these locations, at least in part, as a consequence of simple mechanical factors. Whether there is an additional relationship between tumor cells and nerve elements is not known.

I have discussed elsewhere the prognostic significance of "basement membrane" around invasive aggregates of cancer cells in the cervix *(10)*, and perhaps one can extrapolate from that discussion to other types of cancer. In summary, when the invasive cancer is poorly differentiated, the presence or absence of basement membrane probably has relatively little prognostic significance. When the aggregates of tumor cells are well differentiated, the presence of basement membrane may be of minimal prognostic significance, while the absence of basement membrane may indicate that the host can deal with at least some of the invading clusters of cancer cells.

The significance of inflammatory cell infiltration at either primary or metastatic sites has been studied by many morphologists for a long time. The numerous lymphocytes often associated with medullary carcinoma may be responsible for the relatively good prognosis for patients with breast cancer. In cases of breast cancer, Berg feels that the presence of plasma cells indicates a favorable prognosis *(11)*. In other tumors, however, I do not believe one can say whether the presence of a specific type of inflammatory cell at the primary site is of predictive value. It is very impressive at times to see an intense eosinophilic infiltrate associated with invasive cancer, but the interpretation of this histologic finding is much less clear than the actual finding. Most recently, however, we have been investigating the prognostic significance of lymphocytic infiltration *in the walls of small veins* at the primary tumor site *(12)*. The veins are readily identified in tissue sections stained for elastic tissue. This histologic finding is characteristic of a delayed hypersensitivity reaction and suggests some sort of host response to tumor. Thus far, however, there is no proof that this kind of reaction has occurred.

BLOOD VESSEL INVASION

The prognostic significance of blood vessel invasion at the primary tumor site has been investigated in specially stained tissue sections, though this entity can at times be distinguished in tissue sections stained with the usual hematoxylin and eosin. When cancer cells invade and fill a small vein, the elastic tissue skeleton of the vein appears to be the last part of the structure destroyed. Consequently, an elastic tissue stain such as Verhoeff-van Gieson will reveal cancer cells within a pre-existing venous lumen. An elastic tissue stain can be used in any pathology laboratory which is routinely processing surgical or autopsy material, and its interpretation does not require special training. Indeed, the stain has been used for many years in many laboratories for exactly this purpose.

At the New England Deaconess Hospital, Dr. Shields Warren and Dr. William A. Meissner, together with their associates, have been recording for many years the presence or absence of blood vessel invasion in surgical specimens from cancer cases. The data from colon cancer cases so studied were tabulated by Swinton and Farha and correlated with clinical data *(13)*. Swinton and Farha found that patients with neither blood vessel invasion nor regional lymph node involvement had a 5-year survival of 60%. Patients with blood vessel invasion at the primary site, but no lymph node involvement, had a 5-year survival of 40%. Those with lymph node involvement but without blood vessel invasion had a 5-year survival of 29%, whereas those with both blood vessel invasion and lymph node involvement had a 5-year survival of only 20%. The difference between each of these figures is statistically significant.

A similar relationship between the presence or absence of blood vessel invasion at the primary site and the presence or absence of metastases in regional lymph nodes has been found in our own investigations of certain other tumors, including cancer of the cervix, breast, and bladder. In our material, however, the prognostic significance of blood vessel invasion is most evident in patients with negative regional lymph nodes.

In table 1, we illustrated the prognostic significance of blood vessel invasion, lymph node involvement, and nuclear grade, as seen in 127 cases of breast cancer. In patients with negative lymph nodes, the presence of blood vessel invasion appears to be of prognostic significance, a finding which was noted in series of breast cancer cases reported earlier by Friedell, Betts, and Sommers *(12)* and by Teel and Sommers *(14)*.

The presence of blood vessel invasion in the histologic specimen suggests that the tumor has the *capability* to metastasize via the blood vascular system; it *may* spread. The presence of histologic blood vessel invasion does not, however, tell us if the tumor has "exercised its option" or if circulating tumor cells will grow at some distant site. The presence of tumor in regional lymph nodes indicates that it has the capacity to leave the primary site by some route and, in addition, can grow at a metastatic site. Positive lymph nodes are a biological indicator that the state of the tumor-host relationship is such that tumor can grow in tissue away from the primary site. If there are both lymph node involvement and blood vessel invasion, the tumor would appear to have both the capability of spreading via the blood vascular system and the ability to grow at a metastatic site; obviously the prognosis should be worse in this group.

INDIRECT CANCER CELL-HOST INTERACTION

Rather less research in this area has been done than in the other two areas. The possibility of assessing response to therapy or the presence of metastases by some relatively simple study of a circulating blood cell component is obviously of great interest when tumors themselves are inaccessible. A few such organ or cell responses to the presence of cancer were listed in the outline. It is anticipated that additional studies of these host responses will be forthcoming in the next few years.

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Cellular Prognosis in Cancer of the Cervix[1, 2]

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WE HAVE studied the prognostic implications of 4 cells in cervical cancer. The frequency of malignant cells, histiocytes, and sensitization-response (SR) cells in the vaginal smear and of mast cells in histologic section appears to have significance.

Cells in the pretreatment vaginal smear can be evaluated by differential counts of 100 consecutive benign squamous epithelial cells. Similar differential counts on 100 consecutive epithelial cells and histiocytes give the percent of histiocytes. Another count of epithelial cells and malignant cells yields the percent of malignant cells.

SR cells are benign squamous basal cells with a dense, finely vacuolated basophilic cytoplasm. If 10% or more of the benign epithelial cells are SR cells, SR is "marked"; if 9% or less, it is "poor" *(1, 2)*. In a series of patients from Boston treated radiologically, the 5-year cure rate in those marked SR was 68% (37/54) whereas it was only 18% (17/93) in those with poor SR (table 1). Somewhat astonishing was the fact that the prognosis of SR was different for radiotherapy and surgery. In patients with Stages I and II showing marked SR, 72% were cured by radiation, but only 40% by surgery. In those with poor SR, 26% were cured by radiation and 69% by surgery (table 2). At least part of this difference can be explained by the frequency of lymph node metastases. Positive nodes were found in 67% of patients with marked SR and in only 17% of those with poor SR (table 3). It is well known that the surgical cure rate in patients with negative nodes is much better than in those with positive nodes, *e.g.*, 62 versus 22% in a combined series.

Table 1.—SR in radiotherapy, 5-year cure

Stage	Marked SR	Poor SR
I	9/14, 64%	7/24, 29%
IIa	10/11, 91%	2/10, 20%
IIb	12/18, 67%	7/31, 23%
III	6/9	1/21, 21%
IV	0/2	0/7
	37/54, 68%	17/93, 18%

Table 2.—SR in Stages I and II, cancer of cervix, 5-year cure

	Marked SR	Poor SR
Radiotherapy	19/25, 72%	9/34, 26%
Surgery	12/30, 40%	68/99, 69%

Table 3.—Frequency of positive nodes in relation to stage and SR

Stage	Marked SR	Poor SR	All
I	9/15, 60%	11/71, 15%	20/86, 25%
II	11/15, 73%	6/28, 21%	17/43, 40%
	20/30, 67%	17/99, 17%	37/129, 29%

Histiocytes were seen in vaginal smears. In patients with Stages I, II, or III cancer treated radiologically, the 5-year cure rate was 42% (46/109) in those with fewer than 21% histiocytes and 68% (76/112) in those with more histiocytes *(3)*.

The concentration of malignant cells in the vaginal smear was only roughly correlated with tumor size. It is evident that some other factor influenced desquamation. There was a good correlation between the concentration of malignant cells in the vaginal smear, lymph node metastasis, and 5-year salvage after surgery (table 4). Patients with <1% malignant cells had only 12% positive nodes and a 92% cure rate. Those with >40% malignant cells had 69% positive nodes and a 38% cure rate.

Table 4.—Concentration of malignant cells in the vaginal smear-positive nodes and surgical 5-year cure rate

Malignant cells (%)	Number of cases	Positive nodes	5-Year cure (%)
<1	24	3, 12%	92
1–9	41	11, 27%	73
10–39	20	5, 25%	55
>40	13	9, 69%	38

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[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

The numerical values of these 3 kinds of cells can be combined to form a cellular index (CI). The added percentages of SR cells, histiocytes, and malignant cells yield *(4)* the CI. The CI has been divided to produce 3 groups: low CI: 1–29; mid CI: 30–49; and high CI: 50 or more. In a series of patients treated from 1957–63, the salvage rates in Stage I were strongly influenced by CI and method of treatment (table 5). Those patients with high CI showed a 61% cure rate with surgery and an 80% cure rate with radiation. By contrast, the low-CI patients treated surgically had an 82% cure rate and those treated radiologically, only 45%. In Stage II, the differences were much less.

Table 5.—CI, 5-year cure rate (CI = SR cells + histiocytes + malignant cells)

Treatment	Stage	High	Mid	Low
Surgery				
	I	11/18, 61%	7/10, 70%	100/122, 82%
	II	6/11, 54%	9/14, 64%	34/61, 56%
		17/29, 58%	16/24, 67%	134/183, 73%
Radiation				
	I	53/66, 80%	13/22, 59%	25/56, 45%
	II	91/195, 46%	17/36, 47%	16/44, 36%
		144/261, 44%	30/58, 52%	41/100, 41%

Mast cells are relatively unknown because they are not seen in conventional histologic or cytologic preparations. We studied mast cells in histologic sections after fixation in Carnoy's fluid (ethyl alcohol 60%, chloroform 30%, and acetic acid 10%) stained with toluidine blue. Mast cells are most commonly found in the stroma near invasive cancer *(5)*. Their concentration there is classified as many or few. The 3–6 year survival rates are in table 6; 82% (47/57) of patients wth many mast cells survived as compared with only 40% (33/83) of those with few mast cells. Both radiotherapy and surgery appeared to be favored by these cells. In patients treated with radiation, 75% survived with many mast cells and only 25% with few. In the surgical cases, 95% survived with many and 54% with few.

Table 6.—Mast cells in stroma near tumor in histologic section, 3–6 year survival (excluding intercurrent deaths)

	Mast cells	
	Many	Few
Stage I		
Radiation	8/8	2/5
Surgery	13/13	14/19
Stage II		
Radiation	16/21	9/22
Surgery	7/8	8/18
Stage III		
Radiation	3/5	0/9
Surgery		0/1
Stage IV		
Radiation	0/2	0/8
Surgery		0/1
All stages		
Radiation	27/36, 75%	11/44, 25%
Surgery	20/21, 95%	22/39, 54%
Both	47/57, 82%	33/83, 40%

The concentration of each of these 4 cells has prognostic significance. At first glance these cells have little in common. However, we believe each manifests a different facet of the same process: host defense. SR cells contain high concentrations of γ-globulin and can be elicited at an appropriate interval in animals by application of antigens. Since the time of Metchnikoff, histiocytes have been associated with phagocytosis and other aspects of immunity. We believe that malignant cells desquamate in proportion to host pressure. They are shed from the inner face of the tumor at a rate comparable to that from the vaginal face. This would account for the high rate of positive nodes when many malignant cells are seen in the vaginal smear. However, another factor must function here, because patients with very high proportions of malignant cells have a bad prognosis. In a group of 14 patients with more than 90% malignant cells, only 3 survived 5 years.

The role of mast cells in host defense is not well delineated, but there is no doubt that they are closely related. Mast cells are elicited by antigens. Antigen-antibody reaction will cause them to shed their granules. The mast cell mediates the dramatic manifestations of anaphylaxis.

These 4 cellular phenomena may have some modest usefulness in the management of cervical cancer. However, their theoretical implications are of greater importance, for they may aid in understanding the complex relation between the benign cells of host defense and the malignant cell.

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In Vivo Prediction of Drug Sensitivity With Cancer-Seeking Isotopes[1, 2, 3]

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A BASIC problem in current clinical oncologic practice is the lack of a rapid and accurate method for predicting chemotherapeutic sensitivity of tumor cells in humans. All too often the patient is exposed to needless drug toxicity while several sequential drug trials are carried out. Each of these trials may last 6–10 weeks, further delaying treatment with an effective agent. Thus, attempts have been made to develop a method whereby a drug regimen may be designed which will take into account the biological peculiarities of the specific host and tumor under treatment. One method of achieving such an end is the estimation of the effects on tumor cell metabolism of drugs as evidenced by changes in uptake of radiolabeled substrates, presumably critical to cell energy production and/or cell replication.

Ideally such a system would presuppose the existence of two techniques: 1) a compound which is selectively taken up in tumor cells and is used in their metabolism, and 2) methods for continuously monitoring such an uptake and retention in tumor deposits *in vivo*.

Various methods have been employed to produce selective uptake of isotopically labeled materials in tumors: ^{125}I-labeled amino-acridines *(1)* and tetraphenyl porphine sulfonate *(25)* in Walker 256 carcinoma in the rat, ^{203}Hg-labeled chlormerodrin *(14)* and ^{32}P-labeled sodium phosphate in human brain tumors and in tumors in the human gastrointestinal tract *(20)*, ^{99M}Te labeling in human parotid tumors *(10)*, ^{131}Cs labeling in superficial human tumors *(6, 11)*, and ^{3}H-labeled naphthaquinol *(17)* in experimental tumors. In addition, purified rabbit antiserum to human fibrinogen labeled with ^{131}I *(21, 23)* has been used. Also used have been specific precursors of metabolic functions, such as ^{14}C-dihydroxyphenylalanine *(3)* or 4-(3-dimethylaminopropyl) amino-7-iodoquinoline-^{125}I *(2)* in malignant melanoma, tritiated tryptophan in carcinoid tumors, and ^{131}I-iododeoxyuridine *(18, 19)* in breast cancer and melanoma.

Recent studies have also been concerned with methods for the continuous monitoring of radiolabeled isotopes in tumor cells *(5, 12, 22, 24)*. Stoll and Burch *(22)* used an external isotope detector, whereas other authors used detectors implanted into tumor tissue. We found that adequate fixation of external detectors was impossible and, accordingly, elected to use the latter method.

This report deals with studies of radiolabeled drugs selectively concentrated in tumor cells as well as with the development of a technique for the continuous monitoring of such isotopes.

MATERIALS AND METHODS

Animal studies.—Two types of experiments were performed in Sprague-Dawley rats. The rats weighed approximately 50–150 g and bore 4- to 7-day-old, intramuscularly implanted, Walker 256 carcinoma. In the first type of experiment, tumor, skeletal muscle, bone, heart, and liver tissues were obtained when the rats were killed from 5–120 minutes after intraperitoneal (ip) injection of ^{32}P-labeled sodium phosphate, ^{131}I-labeled serum albumin, or ^{125}I-labeled iododeoxyuridine (IUDR). Tumor tissue was selected to avoid any areas of necrosis. All tissues were then desiccated and counted. In the second type of experiment, miniature (outside diameter 1.5 mm), halogen-quenched, Geiger-Muller detectors (EON Corp. #5107) were implanted into deposits of Walker 256 carcinoma (fig. 1). Positioning of these detectors was fixed by proximal and distal skin sutures and was subsequently checked histologically. Counts taken up

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[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

[3] Supported by Public Health Service grants GRS 27101-0 from the General Research Support Branch of the National Institutes of Health and RR-54 from the General Clinical Research Centers Program of the Division of Research Resources.

in tumor tissue were continuously recorded after ip injection of the above-named isotopes as well as after ip injection of ^{131}I-IUDR. The uptake of radiolabeled drugs was then compared in untreated rats with that seen in rats 8–24 hours after ip administration of 15 mg/kg cyclophosphamide. Tumors were measured grossly and histologically for evidence of drug effects, and animal survival was recorded.

Human studies.—Similar to that described above in the initial animal studies, a technique was developed for implanting miniature Geiger-Muller detectors into superficial tumor deposits in patients with inoperable metastatic cancer (fig. 2). Tumor tissue was biopsied at the time of all probe implants to establish localization within tumor masses. Administered 48 hours after probe implant was either 5–10 μc/kg Na^{32}PO$_4$ intravenously (iv) or 2–5 μc/kg ^{125}I-IUDR iv. A continuous record of radiation in the implanted probe was achieved by use of a Nuclear-Chicago 8700 scaler and specially modified preamplifier with preset timing and an automatic printer-lister. Patients were treated with single iv pulse doses of 15 mg/kg 5-fluorouracil, 15 mg/kg cyclophosphamide, 4.5 mg/kg dimethyltriazenoimadazolecarboxamide, 0.4 mg/kg methotrexate, 25 mcg/kg vincristine, 60 mg/M^2 bis-chloroethyl-nitrosourea, or they were treated with 30 mg fluoxymesterone or 15 mg diethylstilbestrol in single oral doses. All drugs were given in pulse doses beginning 8–72 hours before isotope administration. In a number of patients, several drugs were given in series, allowing 72–100 hours between drug tests for attenuation effects of previously administered drugs.

The characteristic pattern and mean decrement of isotope retention were then demonstrated. The decrement was calculated by estimating the mean change in slope over a period of at least 72 hours and then correcting for physical decay of the isotope. An estimate was then made of the concordance of the pattern of isotope retention with the clinical antitumor effect, if any, of the drug under study.

RESULTS

Animal Studies

Studies involving the sacrifice of animals at serial time intervals are summarized in table 1. Each number represents the mean of 5 rats. There was a marked preferential uptake of ^{32}P into tumor tissue, sevenfold to tenfold that seen in normal muscle (T/M) and 1.5 times that seen in bone (T/B). Similar data were obtained, but are not shown, for normal liver and heart tissue. Maximal selectivity of uptake into tumor was seen 120 minutes after isotope injection, although peak number of counts taken up/mg dry weight tumor tissue was seen between 30 and 90 minutes. Two days after the injection of 15 mg/kg cyclophosphamide, tumor muscle ^{32}P ratios and peak counts/mg dry weight tumor tissue were markedly reduced. Thus, in this cyclophosphamide-sensitive tumor system, a good correlation was seen between inhibition of isotope uptake and tumor sensitivity. Similar experiments (table 2) were done with ^{125}I-labeled serum albumin as a control system. They failed to demonstrate as marked a selectivity of uptake into tumor with either muscle or bone as with reference tissues, and they did not produce as high peak counts/mg dry weight of tumor tissue. Treatment with cyclophosphamide did not change the T/M ratios of uptake as markedly.

TABLE 1.—Na^{32}PO$_4$ uptake in Walker 256 carcinoma

	No treatment			Cyclophosphamide		
Time (minutes)	T/M*	T/B†	T.C.‡ (cpm)	T/M*	T/B†	T.C.‡ (cpm)
30	9.64	1.54	656.0	6.83	1.13	360.2
60	7.64	1.14	525.1	5.25	0.96	370.1
90	8.18	1.04	564.0	5.85	0.64	322.1
120	10.08	1.30	435.6	3.13	0.49	231.7

* Ratio of tumor to muscle uptake.
† Ratio of tumor to bone uptake.
‡ Absolute radioactive count/mg dry weight tumor tissue.

TABLE 2.—^{131}I with labeled serum albumin uptake in Walker 256 carcinoma

	No treatment			Cyclophosphamide		
Time (minutes)	T/M*	T/B†	T.C.‡ (cpm)	T/M*	T/B†	T.C.‡ (cpm)
30	0.84	1.43	49.0			
60	3.11	4.64	222.8	3.02	4.55	226.7
90	4.25	5.63	415.1	2.42	4.58	285.6
120	2.82	3.66	274.0	2.75	3.99	479.4

* Ratio of tumor to muscle uptake.
† Ratio of tumor to bone uptake.
‡ Absolute radioactive count/mg dry weight tumor tissue.

^{125}I-IUDR showed a fourfold concentration of uptake into tumor tissue compared to that in normal tissues, and uptake was inhibited 4 days after cyclophosphamide injection (table 3). A consistent fall in concentration ratios was not seen after cyclophosphamide administration, however.

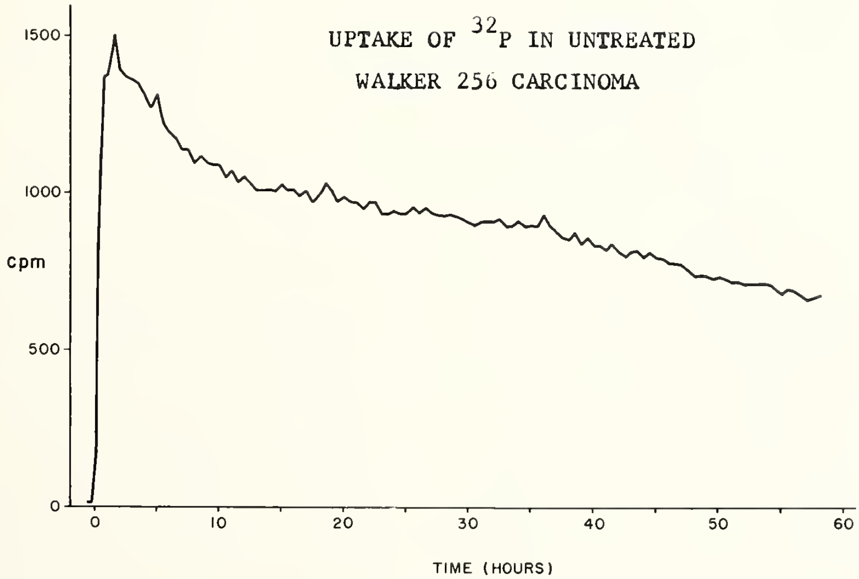
TABLE 3.—^{125}I-iododeoxyuridine uptake in Walker 256 carcinoma

	No treatment			Cyclophosphamide		
Time (minutes)	T/M*	T/B†	T.C.‡ (cpm)	T/M*	T/B†	T.C.‡ (cpm)
30				3.10	4.06	311.4
60	4.27	4.06	543.2	3.67	6.10	424.7
90	4.77	3.76	332.1	3.30	4.34	109.1
120	5.67	4.12	351.2	6.00	4.72	194.8

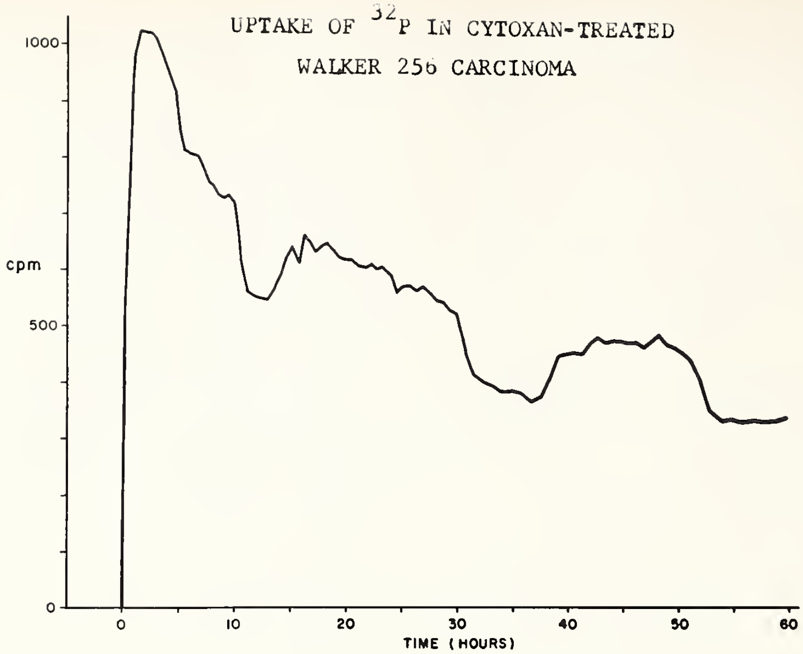
* Ratio of tumor to muscle uptake.
† Ratio of tumor to bone uptake.
‡ Absolute radioactive count/mg dry weight tumor tissue.

When miniature Geiger-Muller tubes were implanted into deposits of Walker 256 tumor, a characteristic curve of ^{32}P (100 μc/kg) uptake and retention was seen. A high peak at ½–1½ hours was consistently observed, followed by a rapid fall of counts measured in tumor, lasting 3–7 hours, and then a further slow decrement of tumor counts for up to 160 hours thereafter (text-fig. 1). A striking qualitative difference was noted in the nature of the curves observed after ^{32}P injection in 2-day cyclophosphamide-treated animals (text-fig. 2). This change occurred after the rapid decremental phase of the curve; it consisted of a cyclical change in counts, varying from 20–50% of the mean counts observed, with a periodicity of 21–27 hours. The periodicity was never observed in rats under identical conditions who had not been treated with cyclophosphamide. Experiments with changing the pattern of the animals' activity and light exposure did not appear to alter the distinctive character of this cyclical effect.

The animal probe data are summarized in table 4. In addition to the striking absence of a cycling of isotope retention in the animals without drug pretreatment, a mean decrement of $<1.1\%$ of initial counts per hour was seen in the untreated animals and $>1.0\%$ per hour in the treated animals. The mean decrements in these two groups were statistically significantly different at $P \leq 0.01$. Normal muscle tissue demonstrated a decrement (following the first 5 hours post isotope labeling), which roughly equaled that attributable to isotope physical decay alone. Because the Walker 256 carcinoma is extremely drug sensitive, all treated tumors demonstrated objective gross and histologic evidence of tumor regression, and concordance of treatment effect and pattern of isotope decrement was seen in all but one untreated animal.



TEXT-FIGURE 1.—Continuous record of counts in miniature detector in Walker 256 solid carcinoma implant in thigh of Sprague-Dawley rat. *Note* initial peak due to blood level (½–1½ hours), fast decremental component (3–7 hours), followed by steady, slow decrement.



Text-figure 2.—Uptake and retention of ^{32}P in Sprague-Dawley rats bearing Walker 256 carcinoma showing cycling effect, with approximately 24-hour periodicity, and amplitude of peaks up to 30% of mean total count rate.

Table 4.—*In vivo* studies in Sprague-Dawley rats bearing Walker 256 carcinoma

Isotope	Number of experiments	Tissue	Drug pretreatment	Mean decrement in isotope retention (per hour)	Concordance of tumor growth (or shrinkage)
^{32}P	4	Muscle	0	0.0015 (no cycling)	
	10	Tumor	0	.0091 (.0075–.011) (no cycling)	9/10
	8	Tumor	Cytoxan 15 mg/kg, ip	.0149 (.010–.022) (24-hour cycling)	8/8
^{125}I-IUDR*	3	Tumor	0	.042 (.031–.062)	
	3	Tumor	Cytoxan (15 mg/kg, ip)	.046 (.036–.065)	

* ^{125}I-iododeoxyuridine.

Human Studies

The ^{125}I-IUDR studies, on the other hand, failed to demonstrate significant difference in either pattern or rate of isotope decrement.

The clinical features of the patients studied with probes implanted into subcutaneous tumor deposits are indicated in table 5. In 3 patients, control probes were implanted into subcutaneous connective tissue sites simultaneously with those in tumor tissue. Melanomas, breast and skin cancer, and sarcomas are the most easily studied tumors because they are superficial. In addition to studies with ^{32}P and ^{125}I-IUDR, some preliminary studies, as yet inconclusive, were carried out with ^{131}CsCl *(6, 11)*.

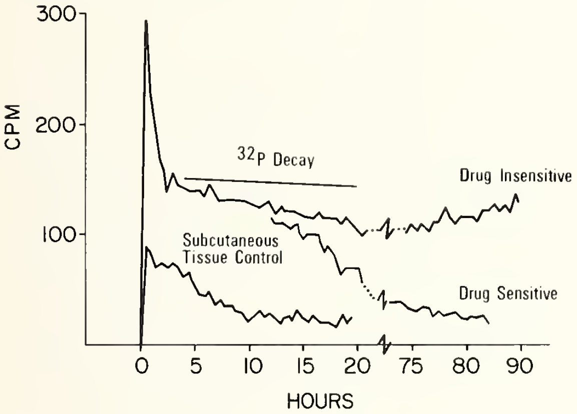
In text-figure 3, the pattern of decrement of isotope retention is summarized in 14 drug trials. No cycling was observed in treated tumors, whether drug sensitive or insensitive. However, a quantitative difference in the rate of decrement of isotope retention was seen between the 2 groups (table 6). The cutoff point between clinically responsive and nonresponsive tumors was approximately 1.0% per hour—strikingly similar to that seen in the animal studies. One patient with melanoma treated with DTIC who evidenced clinical response had a low decrement of retention. However, all other pa-

TABLE 5.—Clinical *in vivo* probe studies

Number	Patient	Isotope	Probe location	Primary site	Drugs*
1	BO	$Na^{32}PO_4$	Tumor, subdermal	Skin appendage	5-FU, CYT, MTX
2	MC	$Na^{32}PO_4$, ^{125}I-IUDR†	Tumor	Breast	EST, AND, VCR
3	EC	$Na^{32}PO_4$	Tumor, subdermal	Melanoma, subcutaneous	DTIC
4	DM	$Na^{32}PO_4$, ^{125}I-IUDR†	Tumor	Melanoma, subcutaneous	DTIC
5	ER	$Na^{32}PO_4$	Tumor	Liposarcoma, subcutaneous	MTX
6	MT	$Na^{32}PO_4$, ^{125}I-IUDR†, ^{131}CsCl	Tumor, subdermal	Breast	CYT, 5-FU, VCR
7	HS	$Na^{32}PO_4$	Tumor	Melanoma, subcutaneous	VCR, BCNU, DTIC

* 5-FU = 5-fluorouracil; CYT = cytoxan; MTX = methotrexate; EST = diethylstilbestrol; AND = fluoxymesterone; VINCR = vincristine; DTIC = dimethyltriazenoimidazolecarboxamide; and BCNU = bis-chloroethyl-nitrosourea.

† ^{125}I-iododeoxyuridine.



TEXT-FIGURE 3.—^{32}P uptake pattern in normal and tumor tissue.

tients had a good correlation with decrements above 1.0% per hour exhibiting clinical response and with those below exhibiting no response.

DISCUSSION

The initial aim of this study was to demonstrate selective uptake of radioisotopes in experimental animal tumor. This aim was achieved with both $Na^{32}PO_4$ and ^{125}I-IUDR. Other investigators have attempted to achieve similar results using a variety of compounds (as summarized above). ^{32}P-containing compounds have been most popular for the continuous monitoring of such uptake because ^{32}P, with its relatively high beta energy, is easily detected *(13, 20, 22)*. The disadvantage of ^{32}P is that it is relatively nonspecific for tumor cells. Although phos-

TABLE 6.—Clinical *in vivo* probe studies with $Na^{32}PO_4$

Number of trials	Primary tumor site	Drugs*	Decrement in isotope retention (per hour): Objective response	Decrement in isotope retention (per hour): No response	Concordance of clinical response: Objective response	Concordance of clinical response: No response
6	Breast					
1		TEST		0.0014		1/1
1		EST		.0051		1/1
1		5-FU		.0023		1/1
1		CYT		.0019		1/1
2		VCR	0.0165, .0245		2/2	
5	Melanoma, subcutaneous					
1		VCR		.0031		1/1
1		BCNU		.0022		1/1
3		DTIC	.0029, .0195		1/2	
1	Liposarcoma, subcutaneous					
1		MTX		.0045		1/1
2	Skin appendage					
1		5-FU	.0215		1/1	
1		CYT		.0075		1/1
Total (or mean) 14			.0151	.0035	4/5	9/9

* TEST = Halotestin; EST = diethylstilbestrol; 5-FU = fluorouracil; CYT = cytoxan; BCNU = bis-chloroethyl-nitrosourea; DTIC = dimethyltriazenoimadazolecarboxamide; and MTX = methotrexate.

phorus is an essential constituent of nucleic acids —compounds which presumably have a high rate of biosynthesis in malignant cells—it is actively involved in intermediary metabolism of the cytoplasmic constituents of the cell as well. Cytoplasmic constituents may be retained in the cell for shorter periods than are nuclear nucleic acids, but they could interfere with specificity of tumor cell uptake, or at least with the direct relation between retained ^{32}P and rate of nucleic acid synthesis or tumor cell proliferation. This obstacle has been overcome by waiting for "steady state" 10–72 hours after isotope administration.

A potentially more specific nucleic acid precursor such as IUDR *(7)* has the disadvantage that its isotopic labels, ^{125}I or ^{131}I, both may penetrate for a significant range and, in probe studies, whether external or internal, both may produce high "background" from adjacent normal tissues. Unfortunately, an ideal label with relative high beta energy that may be coupled with many biochemical substrates has yet to be achieved [*e.g.*, carbon-11 is too short-lived; fluorine-18 has specific affinity for bone only *(15)*].

Interesting parallels may be drawn between our continuous monitoring technique and other recent studies. Stoll and Burch *(22)* have demonstrated a circadian rhythm in ^{32}P retention in untreated human breast cancer. This rhythm corresponded to a similar temperature cycle like that reported by Mansfield *et al. (16)*. Bullen *et al. (5)* also noted cycling in untreated human cancers, in both breast cancer and melanoma, but they noted a periodicity varying from 6–36 hours. In some patients with no cycle, therapy induced cycling, as was the case with our studies of Walker tumor. Bullen *et al.* suggested that the tumor cell is more sensitive at the time of peaking of the ^{32}P retention. They noted that the amplitude of the peak ^{32}P retention (60–350 cpm) varied among patients, as did the amplitude of the cycling (0.1–3.0 × mean cpm). This former point agrees with our studies in humans, although our animal data were more uniform. White *et al. (24)* and Herter *et al. (12)*, however, did not observe marked cycling in human cancers nor did we.

How cyclophosphamide may induce cyclical changes in ^{32}P retention is not defined in these studies. However, it is interesting to speculate that cyclophosphamide does not preferentially affect tumor cells at a specific phase of cell cycle, but the sensitivity of a tumor cell population depends strongly on the fraction of cells in the proliferative state *(4)*. Thus if, for example, preformed DNA were inactivated and cell function in the postsynthetic phase arrested, the remaining viable cells might be synchronized and ^{32}P retention rendered cyclical. An alternative explanation would be related to the induction by drug or tumor sensitivity to an endogenously secreted hormonal material with a diurnal cycle, possibly of pituitary origin.

Another point worthy of note concerns the biological significance of retention of ^{32}P in tumor cells exposed to oncolytic drugs. It is well known that cells may continue to have active metabolic processes after drug therapy, even though their daughter cells may be foredoomed because of incorporation of fraudulent nucleosides or, in the case of alkylating agents, binding or cross-linking of nucleic acid bases or histones. Thus it would seem important, on theoretical grounds, to follow ^{32}P retention through at least one generation time. With the Walker tumor, this may be as short as 1 day, but with human tumors this is certainly as long as 96 hours. Monitoring the cells for at least 100 hours, as we did, would seem wise. In addition, caution in over-enthusiastic interpretation of the results is suggested. Hopefully the development of a beta-labeled nucleic acid substrate for use in these studies might resolve some of the problems with this system.

The development of an *in vivo* method of quantitatively evaluating the rate of nucleic acid synthesis or mitotic activity in the tumor cell requires rapid availability of data, ease of technique, consideration of *in vivo* relation between tumor and host cells, and constant and certain knowledge that the data originated from tumor cells (table 7). Most of these requirements are met by the techniques described. However a number of problems, emphasized by Herter *et al. (12)*, exist which make ready application of this method difficult. Malfunction of equipment, especially the miniature detectors, rendered a significant amount of our data worthless. Restriction to superficial lesions is a serious limitation, although the liver and prostrate are possible internal sites for implantation of detectors. Other problems related to isotope localization, label elution, and tumor affinity need further study. In addition, a final word of caution should be mentioned in respect to the human probe studies. Although good agreement was seen between rates of decrement and clinical response, the number of patients involved in the studies is too small to be conclusive. Furthermore, because most patients failed to respond to drug, the possibility of a sampling error is further amplified.

TABLE 7.—Features of clinical *in vivo* isotope studies with implantable probes

Advantages:
1) Rapid availability of data
2) Ease of technique
3) Reliability of data (vs. *in vitro* studies)
    Tumor cell factors
    Host factors
        Vascular
        Stromal
4) Localization of probe (vs. external detectors)
    Anatomic reliability
    Constant relation to tumor cells
    Reduction of "background"

Disadvantages:
1) Detector, scaler, print-out malfunction
2) Restriction of implant sites (superficial, liver, prostate)
3) Isotope; localization, elution, multiple metabolic affinities
4) Drug action; timing, effectiveness, toxicity
5) Adjacent tissue "background" (e.g., bone, thyroid)

Various techniques may further refine the methods described. Beierwaltes *et al.* (2) used a chloroquine analogue tagged with ^{125}I which appears to have specific affinity for malignant melanoma. Charkes *et al.* (6) demonstrated that ^{131}Cs may have specific affinity for a variety of tumors including lymphoma. Faust *et al.* (8) used oxygenation to enhance localization of radioisotope in brain tumors. These and other methods may make it possible to develop assays for tumor cell sensitivity not unlike those currently available with bacteria. In addition, hopefully, such methods may also be used to estimate the extent and nature of tumor deposits and to deliver cytotoxic doses of radiotherapy to neoplastic cells with radiolabeled drugs.

SUMMARY

Studies on Walker 256 carcinoma have demonstrated a preferential uptake of $\mathrm{Na^{32}PO_4}$ and ^{131}I-serum albumin in tumor over normal tissues in Sprague-Dawley rats. This uptake was maximal at 30–90 minutes. Both the degree of selectivity of uptake of tumor (vs. normal bone, muscle, heart, and liver tissue) and the total counts/mg tumor tissue of ^{32}P were inhibited by treatment with cyclophosphamide, a drug to which this tumor system is known to be sensitive. ^{125}I-IUDR uptake was also suppressed by cyclophosphamide but to a lesser degree than was $\mathrm{Na^{32}PO_4}$. Implantation of miniature Geiger-Muller tubes made possible continuous observations of radiolabeled drug uptake. Cyclophosphamide appeared to produce a cyclical variation and increased decrement, in tumor ^{32}P content, which distinguished the character of the uptake and retention in treated rats from that seen in untreated rats.

No such cyclical changes were seen in treated or untreated human breast or skin cancer, melanoma, or sarcoma. However, a direct correlation was found between the rate of decrement of ^{32}P retention and clinical objective antitumor effects in 14 drug trials.

Although basic problems remain in the concepts and technique, it is hoped that systems such as these may be utilized for estimating extent and nature of tumor deposits, for rapidly predicting drug sensitivity in malignant disease, and for delivering cytotoxic doses of radiotherapy to neoplastic cells with radiolabeled drugs.

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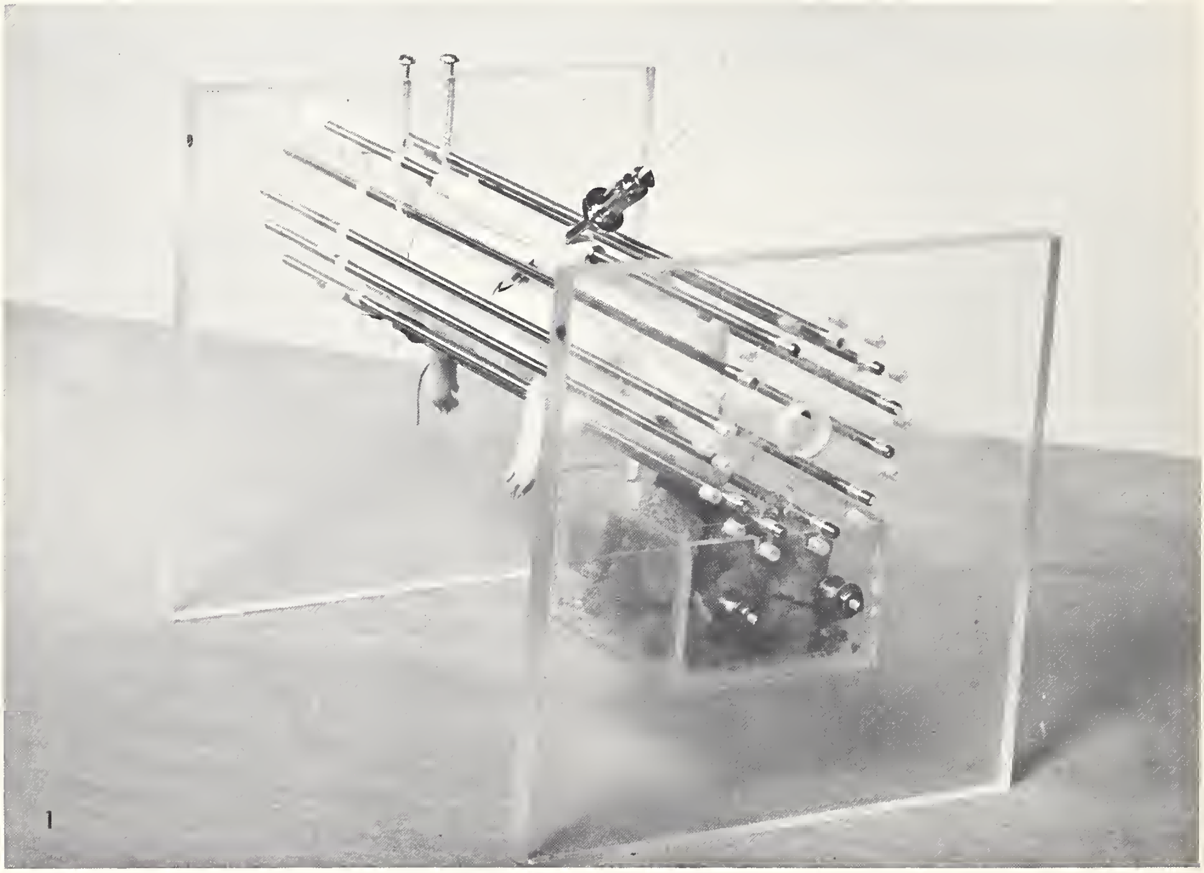

Figure 1.—Experimental rat in cage designed to allow full freedom of movement and freedom to eat and drink. (*Note* hole in front of cage through which water spout fits.) The 12-gauge needle, through which miniature detector is implanted, is shown.

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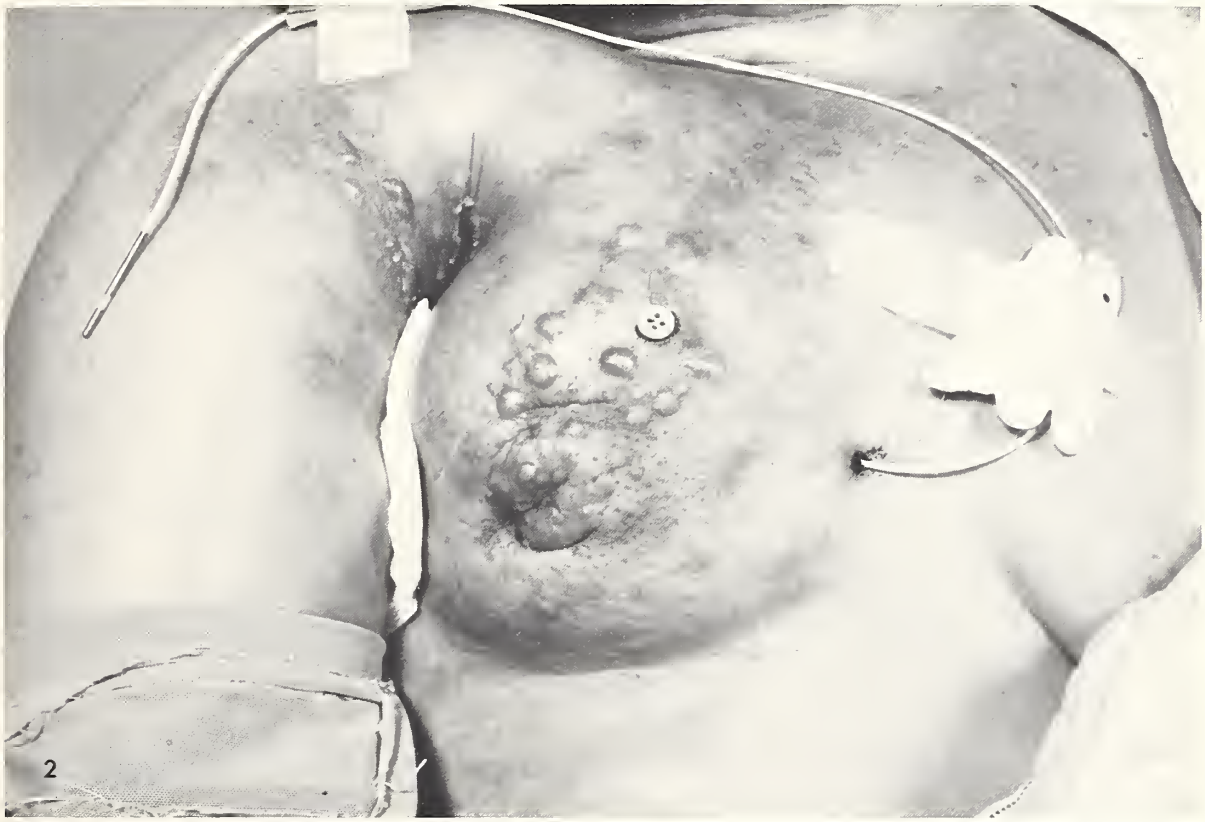

Figure 2.—Miniature detector in a patient with breast cancer. *Note* flexible lead allows easy attachment to radioisotope equipment. Pin connector on proximal end of lead can be attached to, or removed from, preamplifier attachment in seconds.

DISCUSSION

Dr. Cutler: Dr. Graham, since you indicated the comparison of results of 2 treatment methods and you indicated a relationship to the results of certain measurements you took, was there any relationship between your counting of cells and the clinician's decision as to what treatment method to use? Essentially, how do you know that a patient that had a favorable survival with radiation treatment would not have done just as well if he had been treated with surgery?

Dr. Graham: The sensitization-response (SR) data on patients treated with either radiation or surgery were based on clinical judgments, not on cytologic judgments. The same thing was true of the histiocytes and malignant cells. There was some selection in the cellular index group.

Dr. Nathanson: Dr. Friedell, although you mentioned briefly Dr. Nieberg's work on malignancy-associated change, you did not mention your own studies in this regard. Would you comment on the question of nuclear or cytoplasmic changes in normal cells, specifically in the liver, in association with the presence of nonmetastatic cancer in general.

Dr. Friedell: Dr. Nieberg studied the nuclei under very high magnification, usually at about × 1500 or × 1600, for a specific nuclear pattern made up of multiple, vacuolated spaces, which are really spaces between chromocenters. He feels that these spaces indicate or are related to malignancy, but he is not prepared to go further than that at the moment. A group of us have been trying for a couple of years to get common criteria for the statement of malignancy-associated changes. At the moment, I think Dr. Nieberg feels that such changes are recognizable in patients with tumors, but cannot, by any means, be used as a diagnostic test; rather, they should be considered changes present at the moment. I will take this as a jumping-off point to comment on a pathologic test which is very useful in the hands of some people but very difficult for other people to utilize. The pathologist also goes through phases, which are quite comparable to those of the chemotherapist. In phase I, you think you have something of interest, you try to reproduce it in your own material, and eventually you do a double-blind series that seems successful. Then you try to get someone else, utilizing his material, to reduplicate your results. That is the point of hang-up with several studies that seem to be useful only in the hands of a given laboratory or given individuals in a laboratory. This is not to say that the technique is invalid; it just means that the people using the technique initially have not defined the criteria well enough so that another person can reduplicate the technique. This problem has occurred in the past, and at the moment there is a hang-up on "malignancy-associated changes." We get interested in this because, in hamsters and in rats, small transplantable tumors subcutaneously implanted, which do not metastasize, change thymidine labeling of hepatocytes but not the Kupffer's cells. Hepatic parenchymal cells are labeled sixfold in our hamster studies, which suggests a systemic effect of tumor; even a very small tumor does this. In the hamsters, it happens in a week. We think it is different from the other changes that you get with necrotic tissue products.

Dr. Dao: Dr. Friedell, I want to discuss again the heterogeneity of the tumor. About 10 years ago, I studied several hundred slides on breast cancer and tried to predict the response of these cancers to therapy. Finally I came up with a solution: All you have to do is to see how differentiated the tumors are. We found about 75% accuracy. Then we published a paper. But my dream was shattered by a subsequent paper from St. Louis which said that, in breast tumors, heterogeneity cannot be used in prediction. So my question is, in your nuclear gradings, what are your criteria that you can grade at 1, 2, or 3?

Dr. Friedell: There are no criteria. We approached this whole subject very skeptically. A cooperative study we are working on now is set up so that we get the 3 pathologists of the different institutions to agree as to how they will take their material. One important thing is actual measurements. Tumors are rarely measured in many laboratories, surprising as it may seem, but we measured as closely as we could in centimeters and in tenths of centimeters. We took 3 tissue samples if the lesions were less than 3 cm in diameter; we took 5 samples if the lesions were over 3 cm in diameter. The blocks were sent to us. We recut the blocks, stained them with hematoxylin and eosin, an elastic tissue stain, and took some unstained slides for other studies. In each of the slides, different observers attempted to grade nuclei. We expected to see a range from 1–3 in all sections, but we did not. Then we expected to see a range from 1–3 from different parts of the tumor, but we did not. We have over 100 cases so far—too few to make the kind of judgment for which you are asking. So far we do not believe there is much variation—maybe from one grade to another, but not the whole spectrum—in sections from different areas of the tumor. It seems to be fairly consistent. I cannot totally explain it, except to remind you that we were looking at nuclei, not at areas of the tumor that may vary in their differentiation from a glandular pattern to a relatively nonglandular pattern. Thus the same thing is true in blood vessel invasion. We are trying to do reproducible things. We have gone through two workshops in which the slides were mixed up by the statistical group at Louisiana State University. Four of us looked at the unknowns and tried to see if we could approach the same level of quantitation in these morphologic studies as you can get elsewhere. My feeling is that there is not much variation.

Dr. Nickson: Dr. Graham, have you correlated your results with the rating of the histologic material?

Dr. R. Graham: We did a study in Cambridge, England, in which we graded the material as anaplastic, differentiated, and squamous. The only correlations we found were in the mixed and anaplastic tumors.

Dr. Shore: Besides the friendly pathologist, there is also the friendly clinician. In certain instances, it is the experience of the clinician that grading does not correlate well with the prognostic value of clinical staging. This is especially true in carcinomas of the cervix, in which clinical staging is still a very useful prognostic tool. It is probably also true of oral carcinomas of the larynx. Would you like to comment on this, Dr. Friedell?

Dr. Friedell: We try to get several parameters of the tumor. As a pathologist, I am very concerned about the gross, subgross, and microscopic levels. As I pointed out, there is a very distinct correlation between a positive lymph node in cancer of the cervix and the volume of tumor. If the volume is classified stage I and nodes are positive, the 5-year survival is 50%. If the nodes are positive and the volume is classified stage II, the 5-year survival is 15%. In our studies with material from Langdon Parsons in Boston, if the volume was the equivalent of one classified stage III or IV and the node was positive, survival was almost nil. We are very much interested in that. We do not suggest that, in bladder tumors, grading is any substitute for staging. Obviously, the most important thing in the prognosis of bladder tumors is the degree of penetration of the bladder wall. But in a given stage, the grade, I think, will prove to be relevant.

Dr. Concannon: I shall comment on Dr. Graham's report. Suppose you survey the literature on cancer of the cervix and find that the average survival of stage-I carcinoma with surgery versus radiotherapy is about 74%. Now, if you do not look in the surgical institutions and look at the radiotherapy patients who have not had surgery, your 5-year survival in stage I is about 45%. So basically, what you are saying is that the surgical institutions have more of certain cases. Some years ago Professor Kottmaier, a radiation therapist from the Radiumhemmet, Stockholm, Sweden, said that there is no place in this country or anywhere in the world where patients are examined and staged by radiologists and surgeons. The decisions are made on how to treat the patient by a double-blind technique, which is then followed for results. Dr. Graham, is the selectivity so great in the way you chose patients for surgery that it is impossible to say rationally at the end of the period of time that you can relate this back to a retrospective study? The next point that I would like to make is that, right now in this country, there are many radiation therapy series that have reported stage-I five-year survival rates of 90% and stage-II five-year survival rates of 75%. I contend that, if the SR and these other things are so important in survival, then the survival rates should not be as high as they are.

Dr. J. Graham: Your quotation of Kottmaier is probably correct. Indeed there has been no satisfactory series in which the treatment was randomly selected. It is highly desirable that there be such a series. A number of years ago, we inaugurated a series that we thought would solve this problem. Unfortunately the series was ruined by the radiotherapy, for all our initial data had been based on relatively modest doses of radiotherapy. In our study series, we left the radiotherapy in the hands of others, and much larger doses were given, which greatly affected the results. It is evident that the dose rates, or the doses of radiation given, affect the prognosis. Indeed, if you give high doses of external radiation, you salvage a group of patients quite different from those given more modest doses. For example, the Stockholm method of treatment for a stage IIB or stage III case will give the patient a certain total integral dose. The dose used in our hospital was more than 3 times that; although the salvage rates end up in the same percentages, you do not save the same groups of patients. This is a good demonstration of supralethal effects. The salvage rates tend to go down in patients very sensitive to radiation, when they are given a high dose of heavy radiation.

It is quite true that patients in stage I who have been treated with radiation have a 90% salvage rate and other patients who have been treated with surgery also have a 90% salvage rate. These I can only explain on the basis of how exquisitely you select your patients.

Dr. Powers: The selection of the patients that received radiotherapy with a result of 90% survivals, I assume applied to all comers; the result of stage II of 75% survivals applied to all comers. The real question is, since the results of the surgical or radiation therapy appear to reveal low survivals, were those patients selected strictly on the basis of RR–5R, or other of these histological techniques, or were they selected by other means? Were they prospective studies or retrospective studies?

Dr. J. Graham: You can take the same group of cases and by staging them somewhat differently, you can obtain quite different levels of salvage at each stage level and still end up with the same result.

Dr. Hall: Dr. Friedell, you briefly mentioned sinus histiocytosis. Would you elaborate as to whether it means anything at all?

Dr. Friedell: We are trying to establish some meaningful pattern for sinus histiocytosis. At the moment, we cannot get from Dr. Black enough clear criteria to permit us to identify cases of sinus histiocytosis with sufficient reproducibility. We have been trying also to collect all the lymph node material into a central pathology grouping, and the material is available if you want to use it. At the moment, we are not using sinus histiocytosis in the prognostic work.

Dr. Morton: A number of years ago when I was involved in immunotherapy, I can remember running down to the pathologist with a section and saying: "Look at this beautiful lymphocytic infiltration around the tumor; this has to be an immunological response." And the pathologist would say: "Don't pay any attention to that; we see that all the time." It is really very striking how much variation there is among carcinomas in the amount of lymphocytic infiltration around the periphery of the tumors. What does the pathologist think about this amount of infiltration? Has there ever been an attempt to grade this infiltration in some manner? This is not going to be the whole answer, because obviously there are two things here: the growth potential in the tumor and the host response.

Does the amount of lymphocytic infiltration around the tumor indicate how the individual reacts to his tumor? In grading lymph node metastases, there is a tremendous variation between surgical specimens in the number of lymph nodes involved. I have heard stories that, if you take serial sections of lymph nodes from carcinomas of the breast, the instance of positive lymph node metastases will go up with increasing numbers. How can you, in any one case, standardize your procedure to decide whether the patient does really have lymph node metastases or not?

Dr. Friedell: Whether lymphocytic infiltrates mean anything I will avoid for a moment, although many people, including Dr. Louis Thomas, pathologist at the National Cancer Institute, have looked into this matter. If you do an elastic tissue stain, you can identify some lymphocytic infiltrates in the vessel walls, which are a hallmark of a delayed hypersensitivity reaction. If we look for that, it is a little different from looking at the total aggregate of the tumor. In grading lymph node metastases, we are not grading metastases. These are nuclear grades done on the primary tumor. Again, I emphasize that nuclear grading need not be the same as, or have a correlation with, tumor grade. Insofar as the number of metastases you find if you section the nodes, I think Dr. John Pickren showed that it did not make any difference if you missed some of the tiny nodes, for the prognosis was going to be the same. I think Dr. Pickren, pathologist at the Roswell Park Memorial Institute, Buffalo, New York, had a 25% increase in very small nodes and found the prognosis did not change. What I suggested to you here can be done in any pathology laboratory in which tissue is studied. These suggestions are not esoteric and do not require another pathologist added onto the staff.

Dr. J. Graham: Long ago, a method of histologic prognosis was suggested by the Boston surgeons, Dr. Pamberton and Dr. Smithwick. This method related to lymphatic infiltration about cancer of the cervix.

Dr. Nickson: I shall make a general comment about testing predictors. If you are going to try to treat a group of patients predicted to be either good or poor respondors, the first principle is that you have to apply a standard method of treatment with the most uniform technique so that you can test the validity of the predictor. It is equally clear that you cannot have premature feedback of the predictors into the selection of patients for treatment. The ability to test a predictor can be flawed by the unfortunate fact that the probability of a 5-year salvage on patients in some institutions is much lower than that in other centers. Thus there must be internal controls in a study so that you can test a predictor.

Dr. J. Graham: You must have uniform surgery and/or radiotherapy, and you need to maintain it at a constant level for a long period. Unfortunately, in the past decade or so, the amount of radiation has increased steadily. Even in Stockholm, the dose level has increased tremendously, so that it just has not been possible to maintain these things at a constant level. My colleague has some figures on this.

Dr. R. Graham: Of 1,800 cases of primary cervical cancer that I have studied, 1,600 are prospective. When the first paper came out on prognosis, it was quite rapidly repeated and confirmed in Oslo, Stockholm, Gothenberg, and Copenhagen. These series were in centers where radium was the primary treatment. As the years have progressed, we have had cobalt machines. I would like to show you what happens when you have a different method of treatment. The figures Dr. Graham showed you were the original figures for SR, stages I and II, 5-year survival. The same figures were true for radiation response, which is the response of the cell during therapy, where the same differences were found statistically significant in 3 other series. Then we gradually added more and more radiation, with larger fields. The integral dose changed tremendously. Where we had about 3000 rads, we now have 5700 rads. In a randomized series for poor SR and good SR, poor SR now rises to 47% and good SR declines to 55%. The whole difference between these prognostic indexes has become blurred. We are not doing the same experiments. The cure rate here for stage I was 66%, very poor with these large doses of 5700. So if you were going to predict, be sure you are doing the same study all the time and using relatively the same dose.

Dr. Hall: Assuming that we can ignore the possibly valid objections to the sampling technique, the presence of many positive lymph nodes at surgery seems to be related to the high frequency of SR response. If there is a high frequency of SR response, then relative to surgical therapy and radiotherapy, the 5-year survival is not so good with surgery. Dr. Graham (J.), can you relate the kind of thing that you are describing with what Dr. Morton described? Is this related to a state of immunity in the host which is altered in some way, and, hence more node metastases take place and the prognosis is poor? Therefore, these patients would be found by Dr. Morton to be negative. If a patient can have immune responses altered in either direction and they are altered in the direction of having more of an immune response to this tumor, is it better to use surgery?

Dr. J. Graham: In the stage I and II cases, which may be subjected to surgery, you have at least 3 possibilities. 1) If the patients have a low SR, they are not adequately stimulated to respond because the lesion is so modest. 2) If the lesion is more extensive, the patients are stimulated to respond, and they develop SR and, at the same time, lymph node metastases. 3) On the other side is the patient who may have such advanced disease that he can no longer respond, *e.g.*, the patient who Dr. Morton found could not respond to the antigen he administered. It seems to us that patients who do have an SR have tumors sensitive to radiation. This is highly suggestive that lymph node metastases may be controlled better by radiation than by surgery.

Dr. Heidelberger: I am puzzled by several aspects of Dr. Nathanson's report. If you are dealing with ^{32}P, which is highly energetic, and if you are dealing with superficial lesions, then why bother to implant this little detector underneath? Why not just put an ordinary detector of suitable size against the skin? You are not getting more selectivity subcutaneously.

Dr. Nathanson: The reason is that you have to keep the detector in precise relation to the tumor for long periods of time. Whereas this may seem relatively easy to you, it is not so easy. From a practical standpoint, it is extremely difficult to maintain an external probe, in precise relation to an external lesion and exerting pressure on the lesion without disturbing it. For instance, if you take such a probe and fix it with a very tight dressing so that it will not move in relation to the tumor cells, you may be altering the vascular supply and a whole host of other factors. In humans, we have kept the probes in for periods up to 3 weeks, and we have had no infection or local trauma. We have used routine methods of maintaining sterility, as one might with an indwelling intravenous catheter of various sorts.

Dr. Heidelberger: Did I miss how you expect to see prognostic effects from this? What did you learn by watching the slope of the decay?

Dr. Nathanson: The assumption is that relatively early and rapid inhibition of uptake of various materials, including substrates for tumor metabolism, may be achieved in the tumor cell as a result of the effect of the drug, which cannot be observed grossly.

Participant: Dr. Nathanson, you implied that activity or diet may play a role. Does starvation or glucose loading affect the ^{32}P uptake? Also, have you looked at a cytoxan-resistant tumor?

Dr. Nathanson: We have not glucose-loaded our animals to see whether that might affect ^{32}P uptake nor have we given the animals insulin and glucose. We have not looked at cytoxan-resistant tumors, but we have studied the use of agents to which the Walker tumor is much less responsive, *i.e.,* methotrexate. Doses of methotrexate in the Walker tumor did not seem to produce results like those seen with the use of an agent to which the tumor is sensitive.

Dr. Skipper: The doses you gave probably would still be only 99.99% of the cells at a single dose. Were you not in essence working with cells that had already been killed?

Dr. Nathanson: There were two types of data: data obtained from animals killed before and with drug, and probe data in which the animals were studied with and without drug. Without drug, you are speaking of the cyclical effects. The drug was given just before injection of the isotope and after implantation of the probe. When the probe was implanted, a steady state was achieved by allowing at least 48–72 hours to pass. Then the drug was given, and shortly thereafter the isotope was given—a lag of between 8 and 24 hours. What we are measuring is retention of label; we are not really studying uptake of label. It represents the relation between uptake and release. If retention occurs, *i.e.,* if the tumor holds on to the label very well, we are not really measuring the dynamics. We are measuring, unfortunately, the sum of the dynamics, not the individual components.

Dr. Hall: Dr. Nevinny and I did some ^{32}P surface measurements as suggested by Dr. Heidelberger. These measurements showed that you can do this with superficial lesions. Whether you use counts per minute (cpm) or use a ratio of the cpm of tumor tissue versus the normal homologous area on the contralateral side of the body, you do get a therapeutic relationship. We studied breast cancer patients who had testosterone administration. There was evidence of an increased uptake of ^{32}P into tumor nodules during the period of relapse, followed during effective treatment by a fall, and then a slow return, which was repeated over two more relapses and responses. This also worked fairly well for successes and failures on corticoids, radiation therapy, and nitrogen mustard. The main problem with doing it superficially is that a large dose of ^{32}P is needed which gives a background lasting for a week and impairs your ability to give another dose later. If you can give a very small dose and you have a low background, the kinetic rates would be more determinable.

Dr. Nathanson: Dr. Heidelberger, we assume that ^{32}P uptake related to general metabolism of the cytoplasm might be of shorter duration and account for less retention in the cell 72 hours after isotope administration than ^{32}P uptake into nucleotides. The latter might be expected to be somewhat more stable and to be retained by the cells in somewhat more constant fashion. Do you think that assumption is justified in this situation?

Dr. Heidelberger: It probably is.

Dr. Zelen: I agree that it is very dangerous to do long-term prognostic studies in cervical cancer. I want to raise a point about women who come into treatment after being tested by cervical smear programs compared to women coming into treatment because of symptoms. Increasing numbers of women are discovered in such programs, and they are in different stages, with different long-term prognoses than the women with cervical cancer in the older literature.

Staging systems are both good and bad, depending on what you are going to use them for. If you are going to use them for understanding the disease, they might be very bad—especially, since, as many of the pathologists have pointed out, evidence is mounting that the pathology of the primary tumor in breast cancer is quite important. The UICC system for staging does not include this at all. Why not multiply staging factors and make staging systems more useful?

Dr. J. Graham: Cervical smear-detecting programs have profoundly influenced the kind of clinical material found in many clinics. You need to inquire what sort of detection programs and what kind of material they have before you draw any firm conclusions about results. It is hazardous to compare the results from one clinic to another. Clinical staging in cancer of the cervix is really quite useful as a prognostic factor within a clinic. It has some limited usefulness in comparing the results from one clinic to another.

PANEL VIII

IN VITRO PREDICTIVE TESTS

Analysis of an Individual Chemotherapy Assay System[1, 2]

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SCREENING techniques for antitumor agents, which make use of malignant and normal human tissue *in vitro,* allow for many comparisons among agents not possible with animal or microbiological screens. Such screening techniques have been reported with human cell cultures by Eagle and Foley *(1)* and DiPaolo and Moore *(2)* and with minced human tissue in solid agar by Knock *(3)*.

Mammalian cell culture techniques, though less expensive and less laborious, have numerous innate disadvantages which are only partially understood. These disadvantages include extrapolation to the *in vivo* situation where the drug may be so modified, altered, metabolized, or excreted as to render it more or less toxic, or more or less effective than originally observed in the *in vitro* assay. Development of host resistance may also make correlation between *in vitro* and *in vivo* tests difficult and at times unimpressive. DiPaolo and Dowd *(4,5)* showed that compounds of proved clinical value or wide effectiveness in animal tumor systems are inhibitory in tissue culture; the exact conditions of the *in vitro* test may often determine the extent of inhibition caused by any one compound, ranging from inactivity to high inhibition.

Although limited, *in vitro* screening can be an important method for selection of the best antitumor agent for a particular patient at a specific time in the course of his disease. This potential is suggested by three considerations: 1) The material challenged is the patient's own tumor, 2) several agents can be tested simultaneously on the same tissue specimen; and 3) the agents can be simultaneously tested on both the tumor and "normal" tissue of the patient. The last point is of some importance where an agent's therapeutic efficacy is due to its differential toxicity to tumor and normal tissue.

The methods of analysis presented here treat *in vitro* test results as a method for selection of agents from some existing armament to treat the patient's tumor. Thus the test can be regarded as a predictor of the clinical efficacy of agents on a particular tumor in a specific patient. No prediction of similar clinical success or failure for the compound under test on other patient's tumors or on the same patient's tumor at a different developmental stage could be made at this time. Data may be accumulated on drug specificity at a certain site or for a particular histological type of tumor, but this specificity is not a necessary assumption of the test.

Questions which might be asked include: 1) Is the *in vitro* test a good predictor of clinical therapeutic success, and what type of evidence does the test itself provide on this point? 2) Are there any specificities of compounds for tissues from certain sites, or of a particular histology? 3) Do tissues from certain sites show greater than average sensitivity or resistance to the compounds tested? 4) Are certain compounds effective on tissues where few, if any other compounds are also effective? Conversely, are certain compounds effective only when many other compounds are effective?

This paper emphasizes the results of the agar-plate test (fig. 1) on tissue obtained from patients from the Roswell Park Memorial Institute.

MATERIALS AND METHODS

Fresh, sterile, human malignant tissue and, when possible, normal surrounding tissue from the same patient were obtained directly from the operating room. The tissue was minced, and agar-pour-plates of the cell suspension and a basic medium were prepared on 90-mm petri dishes. After the agar surface hardened, dry, 12.7-mm paper discs,

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[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

[3] I thank the staff at Roswell Park Memorial Institute, Buffalo, N.Y., for cooperating in this study and for supplying tissues and diagnoses.

[4] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

each impregnated with a concentration of a different chemotherapeutic agent, were placed on the agar-pour-plates which were refrigerated (4°C) and incubated (37°C). The discs were removed, the plate was covered with a 0.05% solution of methylene blue, a redox agent, and the dye was covered with a glass plate. The dehydrogenase activity of the tumor cells reduced the methylene blue. The area of unreduced dye indicated the inhibition of dehydrogenase activity or cytotoxicity. Inhibition by an agent was reported as positive when the diameter of the unreduced zone was >13 mm. Often when the amount of tissue was limited so that the number of cells that could be obtained was too small to permit cells to be placed in a petri dish, capillary tubes were used. The drug, cells, indicator, and nutrients were mixed together, and the capillary was then filled.

Tissues were obtained from 368 patients. From 139 of these patients, a normal tissue specimen was simultaneously obtained; 88 of the 368 patients were subsequently treated with some chemotherapeutic agent.

The chemotherapeutic compounds tested and the number of tissues on which each was tested are shown in table 1. All drugs were tested at a concentration of 100 μg per disc except for AD which was tested at a concentration of 10 μg.

TABLE 1.—Chemotherapeutic compounds used in *in vitro* test

Code	Chemical	Number of tissues
HN$_2$	2,2′-Dichloro-N-methyldiethylamine hydrochloride	368
TSPA	N,N',N''-Triethylenethiophosphoramide	368
AM	Amethopterin	368
AD	Actinomycin D	368
HN$_2$O	Nitrogen mustard N-oxide	368
6-MP	6-Mercaptopurine	368
5-FUDR	5-Fluorodeoxyuridine	288
AB-103	Benzyl N-bis-(ethylenimido)-phosphorocarbamate	283
5-MU	5-Mercaptouracil	309
5-FU	5-Fluorouracil	80
AB-132	Ethyl bis (2,2-dimethyl-l-aziridinyl-ophosphinyl) carbamate	147

RESULTS

Drug Effect on Normal and Malignant Tissue

The ideal compound would be one which inhibits malignant cells but does not affect nonmaligant cells.

Most agents showed activity against normal tissue only as often as against malignant tissue (table 2). Two exceptions were HN$_2$ and 5-FUDR, effective twice as often on tumor tissue alone as on normal tissue alone. Of those agents considered here, HN$_2$ and 5-FUDR showed the greatest degree of selective toxicity to malignant tissue. Interestingly, they also showed the highest proportion of positive results on all tissues tested.

TABLE 2.—Comparison of *in vitro* test results on normal and tumor tissue from 139 pateints

Chemical*	Normal and tumor tissue	Effective on normal tissue only	Effective on tumor tissue only	Effective on neither
HN$_2$	13	9	21	96
TSPA	1	4	7	125
AM	13	11	11	100
AD	13	11	18	97
HN$_2$O	10	5	9	115
6-MP	3	4	9	123
5-FUDR	11	8	18	65
AB-103	1	1	1	77
5-MU	11	10	10	108
5-FU	9	5	4	19
AB-132	3	0	3	64

* See table 1 for explanation of codes.

Drug Effects at Various Sites

The 368 tissues were classified as to their site of origin with use of the broad classes of malignancies from the International Classification of Diseases. Eleven such site groupings, along with a twelfth residual grouping, were established. Sites were grouped into broader classifications when there were few tissues from that site (table 3).

The overall percentage of tissues on which each agent was effective is given in the final row of table 3. 5-FU and HN$_2$ showed effectiveness on the highest percentage of tissues, 29, followed by 5-FUDR with 27%. TSPA, 6-MP, 5-MU, AB-103, and AB-132 showed significantly less activity (11–14%), while the remaining agents were effective on an intermediate percentage of tissues.

If the overall percentage effective for an agent is compared with the percentage effective at each site, HN$_2$, AM, and AD significantly exceeded their overall percentages for tissues from the buccal cavity and pharynx. AD was higher with tissues from stomach (39 vs. 21%), but HN$_2$O was lower for stomach (6 vs. 23%). AD showed little effect on tissue from large intestine (5 vs. 21%), whereas 5-FUDR had a greater than average effect (63 vs.

TABLE 3.—Drug effectiveness by site of tumor; percent of tissues on which drug was effective

Primary site of tumor	Number of tissues	Chemical*										
		HN_2	TSPA	AM	AD	HN_2O	6-MP	5-FUDR	AB-103	5-MU	5-FU	AB-132
Buccal cavity and pharynx	19	53	16	37	37	16	21	(16)† 37	(15) 20	(17) 18	(3) 0	(10) 10
Stomach	18	34	6	28	39	6	6	(12) 17	(15) 7	(12) 25	(6) 67	(10) 10
Large intestine	39	21	3	16	5	16	10	(19) 63	(35) 6	(37) 5	(20) 35	(25) 16
Rectum	46	24	4	2	18	15	4	(37) 30	(41) 10	(42) 24	(9) 33	(24) 8
Other digestive tract sites	43	32	16	16	21	25	16	(36) 28	(38) 5	(44) 9	(7) 43	(16) 0
Respiratory system	27	33	15	22	19	26	4	(22) 23	(21) 24	(23) 9	(5) 20	(15) 20
Breast	42	22	10	36	24	29	10	(34) 24	(27) 7	(32) 19	(8) 13	(8) 50
Female genitals	18	28	17	11	17	22	22	(18) 22	(8) 25	(11) 0	(0) —	(0) —
Malignant melanoma	15	13	7	13	20	26	13	(13) 31	(11) 9	(14) 21	(16) 17	(6) 0
Bone and connective tissue	38	34	10	21	31	31	10	(37) 35	(24) 17	(29) 3	(1) 100	(1) 0
Lymphatic and hematopoietic sites	13	31	31	0	8	39	8	(11) 0	(9) 0	(9) 11	(2) 0	(2) 50
Other sites	50	32	10	12	20	24	10	(24) 29	(39) 33	(39) 13	(13) 16	(13) 18
Total	368	29	11	18	21	23	11	(288) 27	(283) 14	(309) 13	(80) 29	(147) 14

* See table 1 for explanation of code.

† In last 5 columns percentage is based on number of tissues shown in parentheses.

27%). AM showed little effect on rectal tissue (2 vs. 18%), but a greater than expected effect on breast tissue (36 vs. 18%). Some of these interactions of agent and tissue could be due to chance alone, however, and must be interpreted with caution. Other interesting interactions, though suggestive of drug-site affinity, cannot be considered significant because of the small number of tissues tested from that site; *e.g.,* 5-FU on stomach tissue showed positive effects on 4 of 6 tissues (67%) opposed to 29% overall.

Tissue Susceptibility to Agents

Of the 368 tissues tested, 21% were not affected by any agent tested in the *in vitro* system (table 4). Most tissues (54%) showed response to only 1 or 2 agents, and none responded to all 10 agents tested simultaneously. Tissues from the buccal cavity and pharynx, respiratory sites, and bone and connective tissue showed the greatest *in vitro* susceptibility to at least 1 agent. The lymphatic and hematopoietic sites and the female genital sites were the least susceptible.

TABLE 4.—Percentage of drugs effective on each malignant tissue classified by primary site of tumor

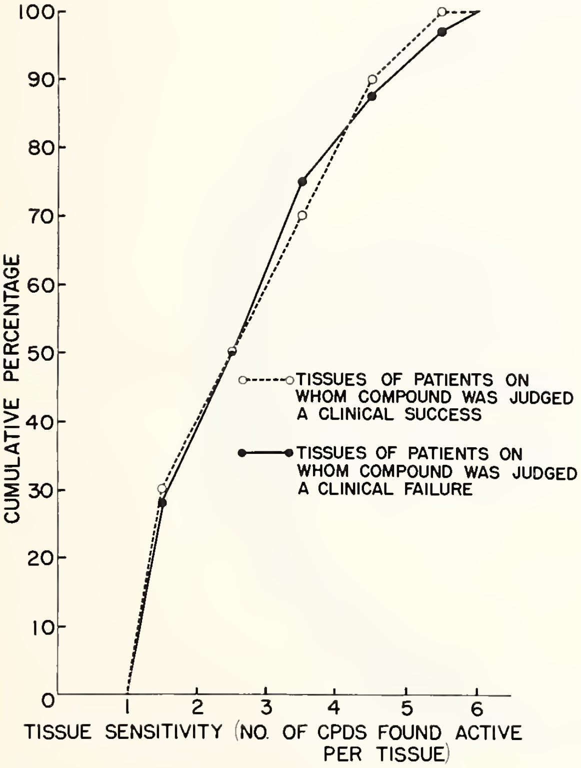
Primary site of tumor	Number of tissues	Number of effective drugs by tissue							
		0	1	2	3	4	5	6	7 or more
Buccal cavity and pharynx	19	0	27*	27	37	5	0	0	4
Digestive organs and peritoneum	146	22	34	23	10	6	1	2	2
Respiratory system	27	4	44	26	7	22	17	0	0
Breast	42	22	38	19	5	7	5	2	2
Female genitals	18	34	34	11	5	11	0	0	5
Malignant melanoma	15	33	40	13	7	7	0	0	0
Bone and connective tissue	38	10	36	29	13	0	3	6	3
Lymphatic and hematopoietic sites	13	39	15	23	15	8	0	0	0
Other sites	50	28	34	14	12	4	8	0	0
Total	368	21	32	22	11	7	2	3	2

* Percent of tissues showing *in vitro* inhibition to test drugs.

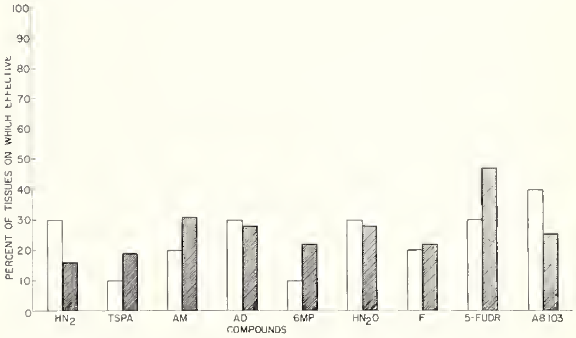
Certain agents also were more effective singly (when no other agent was effective) to a greater degree than expected on the basis of their overall performance. Among these, AM was singly effective *in vitro* on 14 tissues (5 expected), and 5-FUDR was singly effective on 12 tissues (6 expected). Most tissues on which AM was singly effective were from the breast, and most tissues on which 5-FUDR was singly effective were from the buccal cavity and pharynx.

To determine whether tissues from patients on whom a compound showed clinical effectiveness were more sensitive to chemotherapy than tissues from patients who showed no clinical improvement, the frequencies with which 1, 2, 3, or more compounds were effective on tissues from each of the two series were compared (text-fig. 1). The distribution did not differ, indicating that the tissues of patients responding to chemotherapy were no more sensitive, as defined in this paper, than those from patients not responding to chemotherapy.

The percentages of tissue on which a drug was effective for those patients in whom objective improvement was noted and for those in whom objective improvement was not noted were compared for each of the drugs used. Text-figure 2 presents the *in vitro* results obtained by the agar-disc-assay technique. From the small series involved and the distribution noted, it cannot be said that any one drug was more effective in those patients in whom objective improvement was noted.



Text-figure 1.—Comparison of tissue sensitivity from patients who did or did not respond to therapy.



Text-figure 2.—Comparison of drug effectiveness for patients in whom improvement was noted *(open box)* with patients in whom improvement was not noted. *See* table 1 for explanation of chemical codes.

Clinical Correlation With *In Vitro* System

In 89 patients the clinical effectiveness of the agents, as measured by an objective clinical impression (decrease in tumor size or disappearance of ascites), was compared to the *in vitro* results (table 5). Of the 46 patients treated with agents that had been effective on the agar-pour-plate test, 12 had clinical improvement. This is a statistically significant increase in clinical improvement. Of the 47 tissues showing *in vitro* responses, no one agent was more effective on the 12 tissues of patients with clinical improvement than on the 35 patients without clinical improvement. Nor was any site more frequent among the clinical responses than among the nonresponses. Further, the tissues of the 12 patients who showed clinical improvement were not more susceptible in terms of the number of effective compounds per tissue than were the tissues of the 35 patients who had no clinical improvement. Of the 42 tissues showing no *in vitro* responses, 25 patients did not improve with any agent tested. In the remaining 17 patients, an agent other than the one recommended by the agar-pour-plate test (*i.e.*, one which showed up noneffective) was used.

Table 5.—Relationship between effectiveness of drug as predicted by agar-pour-plate method and observed clinical impressions on 89 cases

Objective clinical impression	Agar-pour-plate test		
	Noneffective	Effective	Total
Noneffective	42	35	77
Effective	0	12	12
Total	42	47	89

DISCUSSION AND CONCLUSIONS

This test allows for some indication of differential toxicity to malignant and "normal" tissue. Two agents often effective *in vitro*, HN_2 and 5-FUDR, showed evidence of differential toxicity; another agent effective *in vitro* (5-FU) was effective against "normal" as often as against malignant tissue.

While evidence indicates that some agents perform better than others on tissues from various sites, the recommendation made from the test does not depend on an agent–site specificity. Tumor biopsies from the same patient at different times reflect *in vitro* the clinical changes in the response of tissue to agents. Only three instances of multiple testing and treatment on the same patient at different times were observed.

The test does account for the metabolic activation or inactivation of an agent that might occur in patients or *in vitro*. At this stage of development, it does not provide recommendations as to optimum dosages or optimum therapeutic regimens.

The test may be of value in clinical chemotherapy in indicating the agent of choice for a patient and in determining which patient will not respond to chemotherapy.

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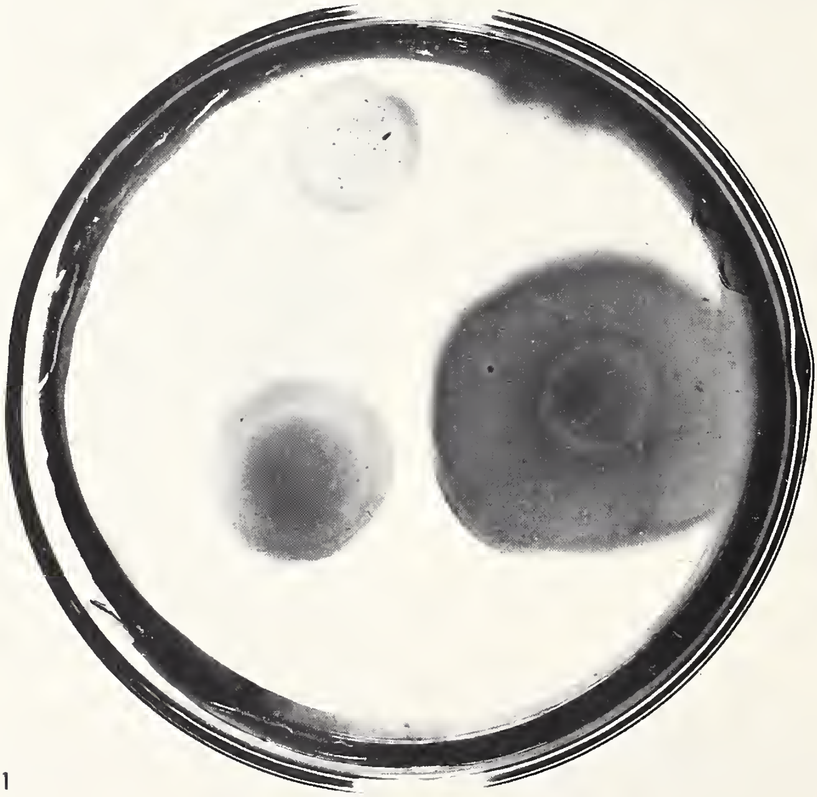

Figure 1.—Agar-pour-plate test, demonstrating zones of inhibition (unreduced methylene blue).

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Qualitative and Quantitative *In Vitro* Sensitivity Tests for Cancer Chemotherapy[1, 2]

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EXTREME CHEMICAL variability among cancers of the same histopathologic type has been noted by many workers. Clinically, the varying response of patients treated with the same drug for the same histologic type of cancer illustrates again the chemical heterogeneity of histologically similar tumors. Clinical cancer chemotherapy is one of the most difficult problems known in the chemical kinetics of competing chemical reactions. One set may lead, for example, to desirable anticancer effect, as by attack at functional groups of cellular polymers of the cancer cells. Another set may lead to undesirable wasting of the drug, as by detoxification in normal or cancer cells, without any anticancer effect. For difficult problems in chemical kinetics, some form of chemical matching of chemical answer to chemical problem appears mandatory.

Our own work on clinical cancer chemotherapy has been guided by four types of *in vitro* sensitivity tests, two qualitative and two quantitative *(1–17)*. The two qualitative tests are: 1) agar plate assay of Miyamura *(18)*, as modified in our laboratories, on cell suspensions from fresh surgical specimens; and 2) histochemical studies of activity of selected sulfhydryl (SH) inhibitors based on block by active SH inhibitors of subsequent staining of protein SH groups by an intense stain for SH groups, an arsenosonaphthyl ethylene diamine derivative.

The two quantitative tests are: 1) the Schrek slide-chamber and time-lapse cinemicrographic studies of drug activity on circulating blood cells of patients with leukemia *(19)* and 2) measurements of drug-induced changes in incorporation of radioactive tracers into DNA, RNA, and proteins, and fractions thereof, of cell suspensions taken from fresh surgical specimens.

A starting point for our work with agar plate assay was the work of Watne and DiPaolo *(20)* who noted a correlation between the agar plate assay of Miyamura and the results of clinical cancer chemotherapy ". . . for a specific tumor, in a specific patient, at a specific time."

Agar plate assay has received most attention in our laboratories *(1–17)* because of its agreement with results of statistically significant drug assays in mice for active and inactive drugs and its agreement with results of clinical cancer chemotherapy when drugs found to be most active from the test are used to treat a patient. Its main disadvantages to us have been its qualitative nature and its inability to provide chemical clues on mechanisms of action of drugs in which we were interested.

Endpoint of agar plate assay is dehydrogenase inhibition, methylene blue being used as indicator. Miyamura suggested research on the media used in the test for more adequate support of the cancer cells. In our tests, we used Eagle's basal medium with essential and nonessential amino acids and vitamins and serum (10% rabbit and 10% fetal bovine for mouse cancers and 20% human, previously heated at 56°C for 30 minutes, for human cancers). Eagle's medium was chosen because this has given us the best generation times in the cytogenerator of Graff *(21)*.

Immediately after removal of the cancer, it was dissected free of normal and necrotic tissue, and minced with scissors and scalpel to a particle size that would pass through a 20-mesh screen. The minced particles in Eagle's medium plus serum and 2% agar were then exposed to drugs on paper discs for 4 hours at 37°C *(9)*. After removal of discs, dehydrogenase indicator was applied, and the results were read by midnight of the day of surgery and 24 hours thereafter *(1–17)*. In all other details, we followed the procedures of DiPaolo and Moore *(22)*.

Categories for grading drug effects were rather

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[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

crude. Drug effects on agar plate assay were graded from 0–2+. *In vivo* effects were graded as + for ≥50% regression in objectively measurable masses, as ± for 25–50% decrease, and as 0 for <25% decrease. Essentially, ± corresponded to slight, but measurable, *in vivo* effect, and + to complete to good regression.

Within the limitations of these very crude categories, agreement of agar plate assay with drug effects in animal cancers for both active and inactive drugs was surprisingly good *(1–17)*.

We treated human cancers only with drugs shown to be most active from the sensitivity tests. Again, within the crude categories set up to grade effects, agar plate assay predictions correlated quite well with results of clinical cancer chemotherapy *(1–17)*.

Although only those drugs were used to treat patients that were shown to be most active from the sensitivity tests, data on test results can still be obtained for inactive drugs by running tests on drugs known to be inactive from the patient's previous history before referral to our program.

In general, negative test results produced by drugs known to be inactive against the patient's own cancer from results of his previous clinical history differed in no way from negative test results produced by drugs inactive against animal tumors *(1–17)*.

In the agar plate sensitivity tests, selected SH inhibitors were frequently more active against animal and human cancers than normal tissues. Histochemical tests were devised, therefore, to study the locus of action of the SH inhibitors and to try to develop more rapid sensitivity tests. The stain used was *N*-[4-(4-arsenosobenzeneazo)-1-naphthyl]-ethylene diamine hydrochloride. The general techniques of Bennett and Watts were followed for the histochemical manipulations *(23)*. Staining of sections of animal and human cancers *(6–11)* resulted from reaction of protein SH groups of the cancer with the arsenoso group of the stain. Nonspecific binding of the stain from reaction of amine groups or Van der Waals forces was ruled out by lack of staining of cancers by analogues of the dye with hydrogen atom or sulfonamide group replacing arsenoso in the stain. Active SH inhibitors reacted with sections of the cancer to make a greater or lesser part of their protein SH groups unavailable for subsequent staining with arsenoso dye. There was a relative decrease in staining results, compared with results in controls. The decrease in staining of treated versus control sections measured the degree of reaction of SH inhibitor with the animal or human cancer. By use of the stain, sections of animal and human cancers were seen to possess a rich network of protein SH groups, not only in the nucleus and cytoplasm but especially in the cell and nuclear membranes and the mitotic apparatus *(6–11)*.

The histochemical test was developed only to the qualitative stage, but the insight resulting from its use led directly to the necessity for our fourth test, a quantitative one, measuring drug-induced changes in incorporation of radioactive tracers into DNA, RNA, protein, and fractions thereof, for cancer and normal cells.

Earlier in our work, however, the necessity for a test divorced from chemical interpretation to check agar plate assay became apparent. Fortunately, available were the slide-chamber and time-lapse cinemicrographic studies of Schrek on drug activity against circulating white cells of patients with leukemia. The endpoint of agar plate assay was depression in dehydrogenase activity. The endpoint of the Schrek test *(19)* was death by morphologic criteria.

In tests run side by side to determine drug effects against circulating blood lymphocytes of patients with chronic lymphatic leukemia, the time-lapse cinemicrographic studies and successive cell counts by the Schrek method corroborated results of the agar plate assay. The time-lapse cinemicrographic studies showed that the SH inhibitor, oxophenarsine, caused death by fixation and rapid death within hours, by contrast with the days required for nitrogen mustard (HN_2) to produce the same effect at comparable dose levels *(1–5, 7, 9)*. Although the Schrek test cannot be used for solid tumors, at a critical stage in our clinical program, it provided encouragement to continue with the sensitivity tests to direct clinical cancer chemotherapy.

The fourth sensitivity test investigated in our laboratories measured drug-induced changes in incorporation of various radioactive tracers into cell fractions: tritiated thymidine to DNA; uridine-2-C^{14} to RNA; and DL-leucine-1-C^{14} to protein. We minced tumor fractions from fresh biopsy specimens in medium 199 plus 10% serum. Samples were incubated with drugs for 6 hours at 37°C and, subsequently, with tracers for 20 hours, by the techniques of Bickis, Henderson, and Quastel *(24)*. Using the method of Kinnory *et al. (25)*, we removed acid-soluble compounds by extraction with cold 10% trichloroacetic acid; lipids were extracted with ethanol-ether; and the residue was boiled with 10% NaCl at neutral or slightly alkaline *p*H to dissociate nucleic acids from proteins, the soluble nucleates finally being precipitated by ethanol. The precipitates were dissolved in formamide, mixed with the phosphor diphenyloxazole, and counted in a Packard 3375 tricarb liquid scintillation spectrometer *(12, 13)*.

Initial studies with animal cancers have shown the expected depression by drugs like 5-fluorouracil (5-FU), HN_2, and methotrexate (MTX) mainly on incorporation of tracer to DNA; action of actinomycin D (AD) mainly on incorporation of tracer to RNA; and the more generalized depression of incorporation of tracers to both nucleic acids and proteins by clinically useful SH inhibitors *(12, 13)*. As expected also, 5-FU, HN_2, MTX, and AD often inhibited incorporation of tracers for normal human leukocytes more than for tumors, whereas clinically useful SH inhibitors often had the opposite effect *(12, 13)*, in accord with clinical data *(1–17)*.

Bickis, Henderson, and Quastel *(24)* noted the generally good correlation between *in vitro* and *in vivo* tumor sensitivities. Our present data support their conclusions. We do not know whether the radioactive tracer studies will supplant the other sensitivity tests for clinical purposes, but certainly the tracer studies will be useful to supplement the other tests in monitoring mechanisms of drug action *(12)*.

For studies on structure-activity relations among homologues and comparisons among classes of drugs, the measurements of drug-induced changes on incorporation of tracers to nucleic acid and protein fractions for normal and cancer cells would appear to be indispensable.

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Prediction of Response of Tumor and Host to Cancer Chemotherapy[1, 2]

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IN 1958 we presented a paper at the meeting of the American College of Surgeons in Chicago *(1)*. At that time, we stated that growth of tumors in patients given toxic cancer chemotherapeutic agents might be accelerated if the chemotherapeutic agent is ineffective against the tumor. We stated further that adverse effects might result from the action of these agents on the host, partly by inhibiting antibody formation. In Japan, I have seen 2 patients with regional perfusion with carcinostatic agents. The first patient, a 48-year-old female, had a malignant melanoma on her left leg, which had been noticed for the first time 3 months before treatment. In July 1961, the lesion was perfused for 1.5 hours with 500 mg of cyclophosphamide, after which the lesion regressed. The patient is apparently free of disease at present. The second patient, a 28-year-old male, had a fibrosarcoma on his right hand; the lesion appeared 1 year before perfusion (fig. 1). The patient's arm was perfused with 2500 mg of cyclophosphamide for 1 hour. A few days later, the lesion began to grow rapidly, and 2 weeks later it had developed into a huge tumor mass about the size of an apple (fig. 2). Because of pain, the arm was amputated. Pathological examination revealed no necrosis of the tumor but demonstrated injury and exfoliation of the endothelium in the arteries and degeneration of the muscle fibers. The importance of having an indication of the effectiveness of carcinostatic agents before they are administered can also be shown in another 2 patients. After these 2 patients, both having adenocarcinoma of the stomach, had been given 40 mg of mitomycin C, one patient had severely damaged cancerous tissue, whereas the other patient had no damage at all.

Although various methods to determine the selection of carcinostatic agents have been reported, little clinical work has been done. The present paper outlines a convenient and quantitative method for clinical use.

First, we developed the succinic dehydrogenase inhibition test, which we call the SDI test *(2)*. We took two steps in our procedure: 1) We obtained a cell suspension of human cancer material, and 2) we observed the activity of succinic dehydrogenase with triphenyl tetrazolium chloride (TTC) to find the effectiveness of carcinostatic agents on tumor cells. To obtain a cell suspension, we designed a simple tissue crusher with many holes, 2–3 mm in diameter. Even hard cancer tissue can be easily fragmented in this crusher with a little pressure. After crushing the tissue, we suspended the fragments in a sterile buffered saline solution. The cell suspension thus obtained was used for the test. After incubating this cell suspension at 37°C for 1 hour with agents in a dilution of 1 : 500 for the daily clinical dosage, we added TTC. We further incubated the mixture for 15 hours. We could observe the color change even after 2 or 3 hours' incubation, but additional incubation was necessary for quantitative determination. Formazan formed from TTC was transferred to the ethylacetate layer. We measured the quantity of formazan by the spectrophotometer calculating inhibition index.

Yoshida sarcoma, ascites hepatoma AH-130, or Ehrlich carcinoma was incubated for 1 hour *in vitro* with a carcinostatic agent. One portion of the suspension was examined for sensitivity; the other portion was transplanted subcutaneously into the mouse. The tumor growth generally paralleled the inhibition index.

The clinical response did not always equal the *in vitro* effectiveness. However, the patients that showed a marked response all had an inhibition index of >75.

Examined by this test were 107 specimens of human cancer. Patients showing an inhibition index of >75 were 70% with the use of nitrogen mustard N-oxide, 38% with the use of mitomycin

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[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

C, 29% with the use of chromomycin, and 18% with the use of cyclophosphamide.

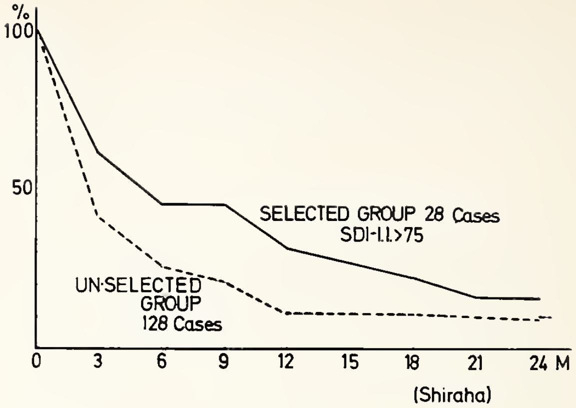
The 18 patients with carcinoma were observed to ascertain the relationship of their sensitivity to drugs and their clinical responses to the drugs. In 15 of these patients, the clinical responses were in agreement with the results of *in vitro* sensitivity, consisting of 11 both-positive and 4 both-negative results. The other 3 patients had positive sensitivity of their specimens, but negative effects were observed clinically after the administration of the drugs, which showed positive sensitivity *in vitro*. There were no patients, however, who had shown negative sensitivity *in vitro* and showed positive effects clinically.

To see the clinical effect objectively and to judge the accuracy of the SDI test, other institutions also performed this test. The accuracy of the SDI test was 89, 67, and 61% in 3 institutions and 82% in our clinic, *i.e.*, an average of 69%, which means 95 cases among 137 showed correct results.

Accuracy of the SDI Test

Kyushu Dental College (Kajiyama)	17/19	89%
Keio University (Suzuki)	18/27	67%
Osaka City University (Shiraha)	42/69	61%
Nagoya University (Kondo)	18/22	82%

Some clinical effects of drugs selected by this SDI test will be discussed on the basis of data from the Osaka City University. In cases of regional perfusion, a selected group of 28 persons with an inhibition index of >75 and an unselected group of 128 cases were compared (text-fig. 1). Compared to the survival rate in the unselected group, the survival rate in the selected group was twice as high, from 9–18 months after surgery.



TEXT-FIGURE 1.—Survival rate after "regional perfusion" with selected drugs.

In 11 cases of arterial infusion and in 114 cases of intravenous administration, we could see some effects in the selected group. When we applied the SDI test to adjuvant use of mitomycin C, among 79 patients with stomach cancer, there were more survivors in the selected group. From 6–12 months after surgery, 20% more survivors were in the selected group compared with the unselected group; however, no difference was found 2 years later (table 1).

TABLE 1.—Survival rate after adjuvant chemotherapy with selected drugs

	Time after surgery (years)						
	½	1	1½	2	3	4	5
Patients treated with selected drugs:							
Number of survivors/No. of patients	34/36	26/35	22/35	16/35	10/31	7/29	3/25
Survival rate (%)	94.4	74.2	62.2	45.7	32.2	24.1	12.0
Patients treated with unselected drugs:							
Number of survivors/No. of patients	31/43	23/40	20/39	19/38	12/35	7/34	5/32
Survival rate (%)	72.1	57.5	51.2	50.0	34.3	20.6	16.5

With a case of arterial infusion of cancer in the upper jaw, the results of the SDI test gave an inhibition index of 92 with 500 mg of nitrogen mustard N-oxide and 83 with 2200 mg of cyclophosphamide. This effect was remarkable, and after 4 years there still is no sign of recurrence of the cancer.

As mentioned above, the accuracy of the SDI test is 69%. To improve the reliability of the SDI test, we developed another simple test, the acid phosphatase deviation test (APD). This test ascertains the effect of chemotherapeutic agents on the activity of one of the lysosomal enzymes (acid phosphatase) in the cell. Acid phosphatase is intimately related to the cell destruction process. We obtained a tumor cell suspension from tumor tissue and divided it among several tubes. After adding the various chemotherapeutic agents, we incubated the tubes at 37°C for 3 hours. We measured the acid phosphatase activity of the supernatant by Lowry's method, calculating the deviation index. We compared the deviation index obtained from Ehrlich carcinoma with the 50% survival of the tumor-bearing animal. We could see the paralleled relation between them. When the SDI test and the APD test were compared in the ascites hepatoma AH-130 of the rat, the inhibition index of the SDI test and the deviation index of the APD test seemed to have the same tendencies.

Applying this APD test clinically, we could predict tumor response very well, if we used enough carcinostatic agents.

As already mentioned, with these simple tests, we could not always predict clinical response for the following two reasons:

1) Distribution of agents in the body and their excretion are not the same, and the concentration of the drug in the tumor differs.

2) Some agents are metabolized in the body. For example, when we applied the SDI test to cultured HeLa cells, the inhibition index was zero with cyclophosphamide. However, when HeLa cells were transplanted into hamster cheek pouches and cyclophosphamide was injected into the hamster, the HeLa tumor responded well to cyclophosphamide.

The effect of various carcinostatic agents on the tumor *in vivo* in tumor-bearing hamsters could be seen pathologically, and the clinical response could be predicted most accurately by the pathologic examination of the tumor.

For comparison, we investigated the effect of carcinostatic agents on the Yoshida sarcoma both *in vivo* and *in vitro*. When the tumor cells were transplanted into hamsters, the magnitude of response (greatest to least) to the various agents was: cyclophosphamide, nitrogen mustard N-oxide, 5-fluorouracil (5-FU), and mitomycin C.

However, when the cell was spinner-cultured, the magnitude of response (greatest to least) to the agents was: nitrogen mustard N-oxide, mitomycin C, 5-FU, and cyclophosphamide. Therefore, cyclophosphamide and 5-FU were more effective *in vivo* than *in vitro*. In the SDI test of the Yoshida sarcoma cell, the order of effectiveness was the same as that in the spinner culture. But, in the APD test of the Yoshida sarcoma cell, 5-FU was less effective than in the SDI test, possibly because the contact time of the tumor cell with the agents is shorter in the APD test. Thus, to make the sensitivity test more accurate, the SDI and APD tests should be performed simultaneously. In clinical cases, when we performed both tests, around half the positive cases were found to be positive in both the SDI and APD tests. Thus, we could obtain more accurate results by performing both of these simple tests.

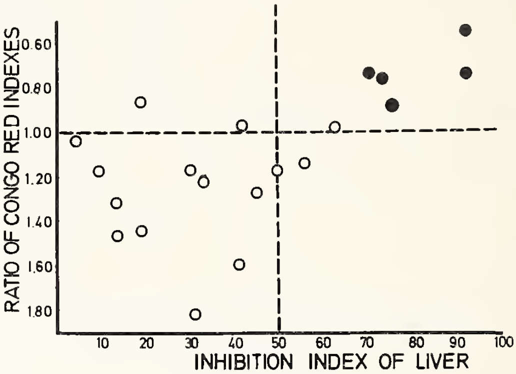
When carcinostatic agents are given to cancer patients, there can be some ill effects, because there is no qualitative difference in the metabolism of the tumor and its host organ. Therefore, a sufficient amount of the most harmless drug should be selected. For this purpose, we tried to predict the side effects of drugs by using the liver as the host tissue. The method was the same as that used in our tumor sensitivity test, except we used liver tissue instead of tumor tissue *(3)*. The results of the liver inhibition index among various agents in clinical cases and the side effects of these agents were investigated. The agents with a liver index >50 all had side effects, such as leukopenia or anorexia, whereas those with a liver index <50 had no side effects.

The function of the reticuloendothelial system was investigated by the method of the Congo Red index to predict the side effects. Cancer patients were examined by the Congo Red index method. The patients were divided into two groups: those with side effects and those without. The patients with side effects had a decreased reticuloendothelial function. Therefore, the side effects can be determined to a certain degree with the Congo Red index. After examining the first Congo Red index, we gave daily doses of the drug twice, just before the third injection of the drug on the 3d day, and then checked the second Congo Red index. The ratio of the first index to the second index within the 48-hour period was "the host resistance index," which shows the effect of the drug on the reticuloendothelial system. We examined the relationship of this index to the side effects. The patients with side effects had a decreased function of the reticuloendothelial system, whereas those without side effects had a somewhat increased function (text-fig. 2). The value of the host resistance index was 0.72 in patients with side effects, 1.27 in patients without side effects, and 1.05 in the control patients (table 2). Thus, we could determine the

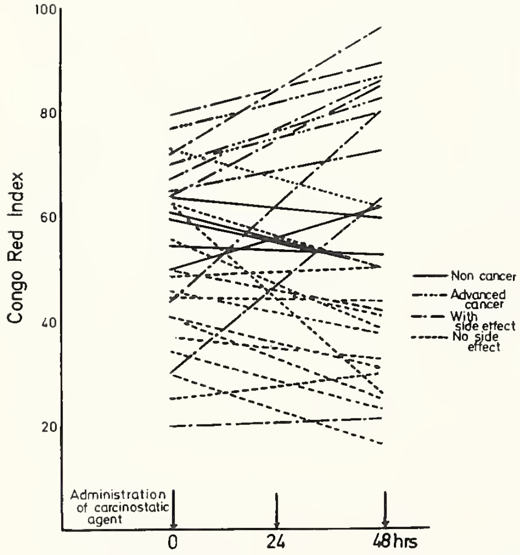
Table 2.—Variation of Congo Red index with and without side effects

Patient	Side effects	Number of patients	First Congo Red index (a)	Second Congo Red index (b)	Hot resistance index, a/b
Without cancer		5	57.9	54.6	1.05
With terminal cancer		4	70.6	81.0	0.87
With cancer	With	7	53.9	75.0	0.72
With cancer	Without	15	46.7	36.5	1.27

side effects after two drug administrations. The relationship of the liver inhibition index to the host resistance index was studied. All patients with side effects had a value of >50 in the liver inhibition index and <1.00 in the host resistance index. Both indexes showed the side effects well (text-fig. 3).



Text-figure 3.—Side effects of drugs in relation to ratio of Congo Red indexes and inhibition index of liver. ● = positive side effects; ○ = negative side effects.



Text-figure 2.—Effect of carcinostatic agent on Congo Red index.

The drugs were classified into four groups according to the SDI test (table 3): 1) those with a tumor index of >75 and a liver index of <50 (col. 1), which means the drug was effective and had no side effects; 2) those with a tumor index of >75 and a liver index of >50 (col. 2), which means the drug was effective but had side effects; 3) those with a tumor index of <75 and a liver index of <50 (col. 3), which means the drug was not effective and had no side effects; and 4) those with a tumor index of <75 and a liver index of >50 (col. 4), which means the drug was not effective and had side effects. In group 4, drugs were only toxic, and sometimes these carcinostatic agents would enhance the growth of a tumor and produce more metastases. The side effects of carcinostatic agents were also investigated in various strains of mice, *i.e.,* dd, C3H, CBA, and C57BL. Mitomycin C was injected twice at a dose of 5 mg/kg. The survival time in days of these mice was 4 in dd, 6 in C3H, 7 in CBA, and 8 in C57BL. Thus, certain drugs differ in their toxicity and side effects among various strains of mice. The liver SDI of these strains of mice was investigated. The value of the inhibition index for dd, C3H, CBA, and C57BL mice was 61, 40, 16, and 11, respectively, which paralleled the survival time. When we examined the Congo Red index of mice of the dd and C57BL strains, the dd strain showed a decreased value in the Congo Red index compared with the C57BL strain which showed a normal value.

The white blood cell count of each strain de-

Table 3.—Inhibition index of cancer and liver (71 cases)

Index of				
Cancer	>75	>75	<75	<75
Liver	<50	>50	<50	>50
Nitrogen mustard N-oxide	10	46	3	12
Cyclophosphamide	17	16	26	12
Mitomycin C	29	22	10	10
Chromomycin	20	22	17	12

creased most remarkably in dd mice, followed by that in C3H, CBA, and C57BL mice.

The SDI index of various organs was also investigated in dd mice.

Liver APD in various strains of mice was investigated and compared with the 50% survival among these mice. With chromomycin, the value of liver APD was in the order of dd, Swiss, C57BL, and CBA; with mitomycin, it was in the order of dd, Swiss, CBA, and C57BL; and with 5-FU, it was in the order of dd, C57BL, Swiss, and CBA. Both results with liver APD and survival were parallel.

Liver SDI and liver APD were compared among various strains of mice. They showed an almost parallel relationship. In cancer chemotherapy, the response is closely related to both tumor and host. To express this point clearly, we calculated the expected effect which might indicate effectiveness more accurately. We transplanted Ehrlich carcinoma into various strains of mice. The tumor APD was expressed as *T* and the liver APD as *L*. If *T* was high, the drug would be more effective; if *L* was high, the drug would be less effective because of decreased host resistance. Therefore,

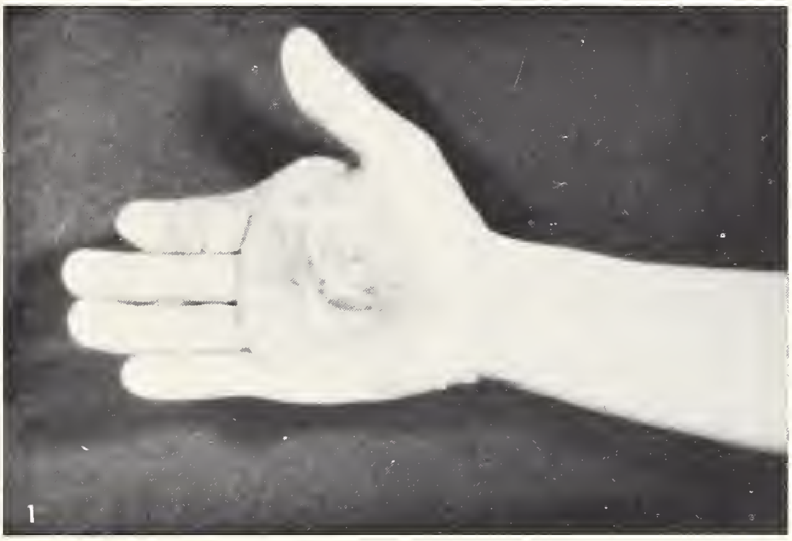


FIGURE 1.—Area of fibrosarcoma before regional perfusion.

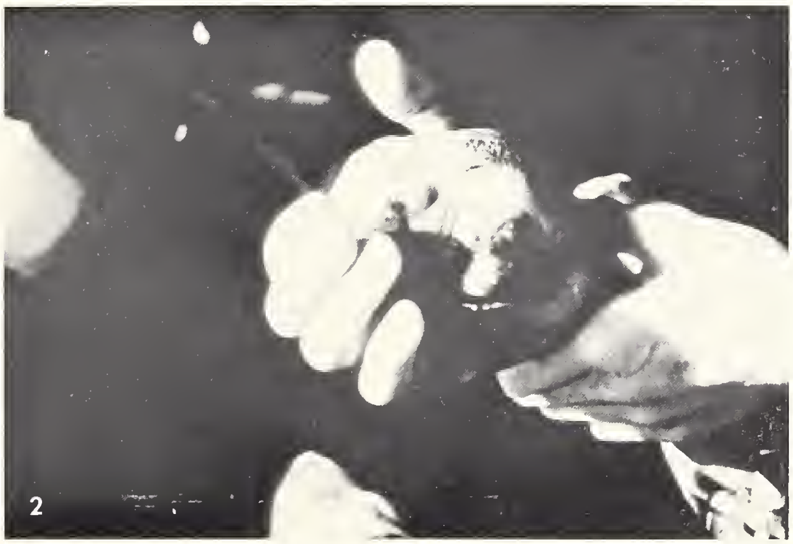


FIGURE 2.—Enlarged area of same patient 2 weeks after regional perfusion with cyclophosphamide.

T — *L* would show the effect of carcinostatic agents on the tumor-bearing animal. When *T* — *L* was high, the tumor-bearing animal lived longer (table 4).

Therefore, in cancer chemotherapy, carcinostatic agents must be selected which are effective against tumors but which, at the same time, have little side effects; this method may aid in this pursuit.

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Table 4.—Host-tumor-drug relationship and tumor APD test and liver APD test in various strains of mice

Carcinostatic agent	Strain of mouse	Tumor APD (Ehrlich ascites cells) (*T*)	Liver APD (host) (*L*)	Expected effect (*T* — *L*)	Survival time (days)
		D.I.*	D.I.*		
Mitomycin C	Swiss	+26.9	+0.9	+26.0	11
	dd		+15.2	+11.7	5
	CBA		—4.6	+31.5	13
	C57BL		—7.6	+34.5	14
Cyclophosphamide	Swiss	+2.4	—2.5	+4.9	3
	dd		+16.2	—13.8	2
	CBA		—3.2	+5.6	3
	C57BL		—9.1	+11.5	5
Chromomycin	Swiss	—8.9	+10.4	—19.3	1
	dd		+14.2	—23.1	1
	CBA		—0.7	—8.2	3
	C57BL		—0.6	—8.3	3
5-Fluorouracil	Swiss	+14.8	—3.6	+18.4	8
	dd		—0.7	+15.4	7
	CBA		—5.4	+20.2	10
	C57BL		—2.4	+17.2	13

* Deviation index.

Prediction of Response to L-Asparaginase Therapy[1, 2, 3]

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AS IT became apparent from studies with experimental animals that L-asparaginase offered interesting possibilities for therapeutic control of certain types of malignant disease, the limited availability of the enzyme imposed a sense of urgency on investigators to find a method offering a rational basis for selection of patients for therapeutic trials. The possibility arises for exploiting several different test procedures derived from investigations with experimental systems. These procedures may be divided into two general categories: 1) tissue culture techniques and 2) asparagine synthetase (ASase) determinations. The tissue culture techniques have been used to determine dependence of the malignant cells on asparagine concentration for growth or for incorporation of small precursors into cellular macromolecules. Alternatively, direct measurement of the ability of the cells to make their own asparagine has been made by assaying their ASase activity; this enzyme catalyzes asparagine formation by the following reaction:

$$\text{Aspartate} + \text{glutamine (or } NH_4Cl) + \text{ATP[5]} \xrightarrow{\text{ASase, } Mg^{+2}} \text{Asparagine} + \text{glutamate} + \text{AMP[5]} + PP_i\text{[5]}$$

This report evaluates the current status of these procedures with respect to predicting the outcome of therapeutic trials of asparaginase in patients having leukemia or solid tumors.

MATERIALS AND METHODS

^{14}C-valine uptake.—The procedure used is similar to that of Sobin and Kidd *(1)*. A basal medium was prepared from 100-ml spinner modified Eagle's medium (Difco 5836), 7-ml horse serum, and 1.5-ml 5% glutamine. Ascites cells of mouse lymphoma 6C3HED were removed from the peritoneum of C3H mice and were washed twice in this basal medium. The cell suspension was adjusted to a count of 2 × 10^6/ml of basal medium. To a test tube were added 3.0-ml cell suspension, 1.5-ml ^{14}C-valine (1.5 μCi) in Eagle's medium, and 1.5 ml of 1) L-asparagine (30.0 μg in Eagle's medium), 2) Eagle's medium, or 3) *Escherichia coli* L-asparaginase (6 IU, Merck, Sharp & Dohme) diluted from a stock solution with Eagle's medium. Aliquots (2 ml) of the 6-ml mixture were placed in separate tubes, and the determination was conducted in triplicate. The preparations were incubated in a 37°C water bath for 4 hours. The mixture was then centrifuged, and the sediment was washed with Eagle's medium, twice with 2-ml 10% trichloroacetic acid, and once with 2-ml ethanol. The precipitate was dissolved in 2-ml hyamine hydroxide at 37°C and was then taken up in 8 ml toluene solution of 2,5-diphenyloxazole and 1,4-bis[2-(5-phenyloxazoly)]benzene (PPO-POPOP) for liquid scintillation counting.

ASase.—Tissue samples were obtained from patients undergoing elective surgery. When possible, the tissue beyond the borders of a tumor being excised was used as a nonmalignant specimen for comparing ASase activity. Samples were generally brought to the laboratory within 1 hour of excision. Homogenates of the tissue were most often spun in a Sorvall Model RC2-B refrigerated centrifuge at a maximum force of 41,000 × *g* for 1 hour, although in some cases they were spun in a Spinco Model L ultracentrifuge at 105,000 × *g*. The tissue extraction procedure and the ASase assay, which was derived from the studies of Patterson and Orr *(2)*, have been detailed previously *(3)*.

RESULTS AND DISCUSSION

ASase

In this laboratory, studies on the mechanism of

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[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

[3] Supported by Public Health Service grant CA 11113 from the National Cancer Institute and by grant 1-334 from the Robert A. Welch Foundation.

[4] I thank members of the surgical staff of the University of Texas Southwestern Medical School for their aid in acquiring surgical specimens, Dr. Robert F. Jones and Dr. Paul C. Peters for their continuing interest, and Ina Derr, Jefferson Janes, Joann Wirsig, and Louise Rothberg for excellent technical assistance.

[5] ATP = adenosine triphosphate, AMP = adenosine monophosphate, and PP_i = inorganic pyrophosphate.

the antitumor activity of asparaginase led to an investigation of the predictive value of ASase measurements. The high degree of specificity of of asparaginase focuses attention, in any consideration of its mechanism of action, on its ability to effect hydrolytic cleavage of asparagine. The hypothesis that has guided our work is simply that those malignant cells with adequate synthetic capacity for asparagine to supply the needs of the cell are resistant to the action of asparaginase and, conversely, those lacking such ability are susceptible to the action of asparaginase. Thus ASase which provides the only biosynthetic pathway for asparagine known in mammalian cells, becomes the key enzyme determining sensitivity or resistance. However, the intracellular events associated with cell death as a result of asparagine deprivation are not clear. In table 1, ASase values are given for a number of resistant and sensitive tumors previously studied *(4, 5)*. As activities are quite low, the micro-enzyme unit was used, *i.e.*, the amount of enzyme producing 10^{-6} μmoles of asparagine/minute. It is readily apparent that the resistant lymphomas all contained greater activity than those susceptible to asparaginase action. These data are in substantial agreement with those presented by Horowitz *et al.* *(6)* for a series of asparaginase-sensitive and asparaginase-resistant leukemias and lymphomas in mice. In their series of susceptible tumors, specific activity of ASase ranged from 0–15 microunits/mg protein, and in their series of resistant tumors from 38–2600 microunits/mg protein. In neither series was there any overlap of values for the resistant and susceptible tumors. Data presented by Patterson and Orr *(2)* for several rat tumors also agree in principle, in that higher ASase activity was found in tumor variants not requiring asparagine for growth in tissue culture.

Table 1.—ASase in mouse lymphomas

Mouse lymphomas	ASase (microunits/mg protein)
Asparaginase resistant	
6C3HED-R	180
P1798-R	170
P388	180
AKR	50
Asparaginase sensitive	
6C3HED-S	0
P1798-S	20

Superficially, these data seem to suggest that the most favorable tumors in man for therapeutic trials of asparaginase would be those lacking ASase, especially when the corresponding normal tissue possessed the enzyme. Such a finding would suggest that the neoplastic transformation was attended by elimination or suppression of expression of the gene for ASase synthesis. With this possibility in view, a number of malignant and nonmalignant human tissues were screened for ASase activity. In most specimens, the specific ASase activity was <10 microunits/mg protein (table 2). In the face of such low values for most nonmalignant specimens, it becomes difficult to reach a conclusion concerning possible sensitivity of a tumor to asparaginase on the basis of its exhibiting little ASase activity. Haskell and Canellos *(7)* reached the same conclusion from studying 20 patients with acute leukemia and 8 with solid tumors. The only group of determinations showing the suggestive pattern described above are those for kidney; 11 of 18 nonmalignant kidney specimens showed significant ASase activity, whereas only 2 of 10 of the renal cell carcinomas showed such activity. Eight of the studies were on paired specimens from malignant and nonmalignant portions of the same kidney, and in only 1 of these studies was the activity higher in the cancer

The mean and median ASase values for the kidney samples recorded in table 3 show the nonmalignant specimens to have significantly greater activity. Because one of these preparations had 10 times (1800 microunits/mg protein) the activity of any other sample, there was some doubt of its validity, and the mean was calculated both with and without that specimen included. Two of the patients with renal cell carcinoma, whose tumor had no detectable ASase, were treated with asparaginase at dose levels of 200–500 U/kg body weight/day *(8)*. The first patient, nearly terminal when therapy was initiated, survived 1 week; the second patient was treated for a period of 6 weeks until the asparaginase supply was exhausted. Asparaginase half-life in the circulation varied between 14 and 26 hours, with concentrations ranging from about 2 U/ml plasma up to 20 U/ml as the dose level increased. Little drug toxicity was found, but no real benefit to the patient could be ascribed to treatment. The only objective evidence of an effect of the asparaginase was a transient increase in urinary uric acid, suggestive of tissue destruction.

Table 2.—ASase in human surgical specimens

Specimen	Nonmalignant (NM) or malignant (M)	Total No.	Number of specimens having ASase (microunits/mg protein) <10	10–50	>50
Bone marrow	NM	3	3	0	0
Breast	M	7	5	1	1
	NM	6	6	0	0
Colon	M	4	2	2	0
	NM	3	3	0	0
Kidney	M	10	8	2	0
	NM	18	7	4	7
Liver	NM	1	0	1	0
Lymph node	M	2	0	1	1
	NM	1	1	0	0
Ovary	M	1	1	0	0
Prostate	M	5	5	0	0
	NM	9	9	0	0
Sarcomas	M	4	4	0	0
	NM	3	2	1	0
Skin	M	1	1	0	0
Squamous cell	M	5	5	0	0
	NM	3	3	0	0
Stomach	NM	2	2	0	0
Testis	M	1	1	0	0
	NM	3	3	0	0
Thyroid	M	1	1	0	0
	NM	6	4	0	2
Uterus	M	1	0	0	1
	NM	3	3	0	0

Tissue Culture Test

This test used by several groups to predict therapeutic response was first suggested by Sobin and Kidd *(1)*. Working with sensitive and resistant forms of 6C3HED, these investigators showed that there was a significant difference in the ability of the two cell lines to incorporate ^{14}C-valine into protein, depending on whether asparagine was present in the culture medium. Sensitive cells incorporated valine only poorly in the absence of asparagine, whereas resistant cells incorporated valine independent of asparagine concentration. This procedure has been used with only minor modification by several groups in conjunction with clinical trials of asparaginase; either labeled valine incorporation into protein or labeled uridine into nucleic acid was used as an indicator of asparagine dependence. Apparently data obtained with the two precursors are parallel *(9)*. In some studies an additional test with asparaginase in the medium was included. Some typical data from our laboratory with mouse lymphoma 6C3HED are shown in table 4. Whereas valine uptake by 6C3HED-R showed little dependency on asparagine concentration or little effect from the presence of 1.0 IU of asparaginase/

Table 3.—ASase in human kidney

	Malignant		Nonmalignant	
	Number	ASase (microunits/mg protein)	Number	ASase (microunits/mg protein)
Median	10	0	18	21
Mean	10	3	17	50
			18	147
Range	10	0–14	17	0–180
			18	0–1800

ml, the uptake by 6C3HED-S was more than 50% inhibited. ASase activity for the sensitive and resistant tumor lines is also presented to show the correlation between the two test procedures.

TABLE 4.—Correlation of valine uptake test and ASase measurements for 6C3HED mouse lymphoma

Lyphoma	Inhibition of valine incorporation (%)		ASase (microunits/mg protein)
	No asparagine	Asparaginase	
6C3HED-R	—4	7	150
6C3HED-S	52	59	0

Oettgen and Schulten *(10)* reported that the bone marrow of 76% of patients with acute lymphocytic leukemia (ALL) showed asparagine dependence by this test, and their observed remission rate of 60% fell only a little short of this figure. For 26 ALL patients with adequate treatment, the test predicted the remission rate accurately in 80–85% of the cases. In acute granulocytic leukemia (AGL), the test correctly predicted the outcome of therapy in 4 cases that were adequately treated. With solid tumors, the correlation was not good, and it was especially poor with malignant melanoma, for which a therapeutic response was observed in only 1 of 9 cases (11%) of a predicted 56%. The results of the use of the valine incorporation test by Hill *et al. (11)* generally agree with those of Oettgen and Schulten for patients with acute leukemia. Their predicted response rate for ALL patients was 80%; 78% (7 of 9) adequately treated patients obtained bone marrow remissions; 6 of the 7 received daily asparaginase doses exceeding 1000 IU/kg/day. Their predicted response rate for AGL patients was 40%, and the observed rate of complete bone marrow remission was 24%. At dose levels above 2000 IU/kg/day, remissions were observed in 4 of 8 AGL patients; unfortunately, no *in vitro* tests were performed on those attaining remission. Ho *et al. (12)* determined asparagine dependency of bone marrow by taking the difference between uridine uptake in the presence of asparagine and uptake in the presence of asparaginase. In their test system, nearly all marrows, normal and leukemic alike, were inhibited as the asparaginase concentration in the medium increased. They concluded that the *in vitro* test failed to correlate with either the magnitude of the initial antileukemic effect or with the production of remission. One problem with interpretation of data obtained with asparaginase in the medium may stem from the glutaminase activity of *E. coli* asparaginase. Thus, if the medium were depleted of glutamine which is required by essentially all mammalian cells in tissue culture, the high rate of inhibition observed by these investigators might be accounted for in part.

Additional Considerations to Account for Discrepancies Between Therapeutic Results and *In Vitro* Tests

One is faced with the problem of accounting for therapeutic failure in renal cell carcinoma and malignant melanoma under circumstances appearing to be highly favorable for success. Several factors may be considered. The first of these involves the effect of asparaginase treatment on ASase activity in the tumor. Prager and Bachynsky *(4, 5)* noted that asparaginase-resistant mouse lymphomas had highly significant increases in ASase after asparaginase treatment of the tumor-bearing animal. A more moderate effect also was noted for certain normal tissues with no demonstrable ASase prior to treatment, and even sensitive lymphomas had a small transitory increase in ASase activity. Broome *(13)* showed that asparagine levels fell rapidly and precipitously following asparaginase administration. It thus appears that, as the exogenous supply is diminished, resistant tumor cells and some normal tissues have the potential of meeting their requirement of the needed metabolite by an increased rate of synthesis. Such observations form the basis of a reasonable mechanism of resistance and, although increasing ASase might account for lack of sensitivity of renal cell carcinoma and malignant melanoma to asparaginase, no specific data are available on this point. Ho *et al. (12)* observed increases in ASase in human bone marrow from 0 to 60–130 microunits/mg protein following asparaginase treatment. One set of measurements was from a cancer patient with an uninvolved marrow, and the other was from a patient with ALL. Haskell and Canellos *(14)* found no difference in initial ASase in leukemia patients responding to asparaginase and in those who did not. Very significantly, however, they observed no increase in ASase during treatment in responsive patients while there was a mean fivefold increase in resistant patients. It is apparent that, while such serial ASase determinations can be used as an indicator of developing resistance, they have no value for predicting initial response.

One might also consider the possibility that the presence of tumors, imposing, as they do, an increased metabolic demand on the host, might elicit an increase in ASase in a number of organs and that the increased biosynthesis might provide asparagine to help maintain the tumor. Patterson and Orr *(15)* observed such an effect on rat liver ASase in animals bearing the Jensen sarcoma, Walker carcinoma, and nutritional variants not requiring exogenous asparagine. However, as long as a patient was under treatment and asparaginase was present, it seems unlikely that enough asparagine could reach the tumor via the circulation to have a positive effect on its survival *(13)*.

An intriguing possibility for transfer of metabolites from one cell to another without passing into the circulation or into the interstitial fluid comes from consideration of the phenomenon of tight- or low-resistance junctions. Loewenstein and Kanno *(16)* demonstrated low-resistance junctions in salivary glands of Drosophila and observed that fluorescein sodium injected into one of the cells could pass readily to another cell of the gland without being detected in the exterior fluid. Furshpan and Potter *(17)* reported that several virus-transformed cell lines exhibited coupling with each other and with the untransformed parent strain. However, not all malignant cells demonstrate coupling, as was pointed out by Loewenstein and Kanno *(18)* in studies with liver and hepatomas; they failed to detect coupling between cells of the tumor, although the normal liver cells were well coupled. Using a somewhat different technique, Subak-Sharpe, Bürk, and Pitts *(19)* observed an interesting metabolic cooperation between a variant of hamster BHK-21 cells lacking inosinic pyrophosphorylase and those possessing the enzyme. The deficient cells could not incorporate hypoxanthine into nucleic acid; but, when they were in direct contact (tight junction) with cells having the enzyme, incorporation proceeded. This observation suggests that something is passed to the deficient cells, although the nature of the substance is unknown. Asparagine might similarly be passed from, for example, normal kidney into tumor cells incapable of making asparagine. The presence of asparaginase in the circulation would have no effect on such a transfer of metabolite.

Finally, the development of antibody to L-asparaginase militates against a therapeutic response with tumors known to be susceptible. C3H mice immunized with *E. coli* asparaginase became resistant to asparaginase treatment of sensitive 6C3HED *(20)*. The presence of antibody was demonstrated by inhibition of asparaginase by immune mouse serum and by the formation of precipitation lines in gel-diffusion experiments. Similar resistance to therapy was noted by Vadlamudi *et al. (21)* for L5178Y leukemia in CDF1 mice that were pretreated with asparaginase.

SUMMARY

The best correlation between an *in vitro* predictive test and clinical response to asparaginase has been observed with the valine (or uridine) uptake procedure in acute leukemia. However, the results have not been uniformly encouraging for all groups using the test, nor has there been any correlation for solid tumors. ASase determinations, while correlating well with response in experimental systems, have fallen short of expectation in clinical situations. Although probably not useful for predicting initial response, when serial ASase assays are performed during therapy, increasing biosynthetic activity may be taken as an indicator of resistance. Other possible reasons for resistance to asparaginase in what may appear to be biochemically favorable situations include: 1) stimulation of ASase activity in normal tissues which are then capable of supplying the tumor with asparagine via the circulation or by coupling at low-resistance junctions or 2) inhibition of asparaginase action by the formation of circulating antibody in the host.

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DISCUSSION

Chairman: Dr. Prager's results with the kidney are, of course, disappointing. Was he measuring the kidney level, measuring cortical or medullary portions of the kidney? It may well be that he may not really have been looking at the aspargine synthetase activity that he wanted to be looking at.

Dr. Prager: In most instances we had mixed cell types in the kidney specimens.

Dr. Hall: Dr. Zelen, is there an element of self-fulfilling prophecy for the solid tumors because failures will occur more commonly in solid tumors with the drugs that we currently have? If there is the likelihood of failure occurring 75% of the time, is it not more logical that a test will predict failures more often, because failures occur more often?

Dr. Zelen: Yes, if one wants to evaluate predictive systems, one has to evaluate the systems in the light of the difficulty of making the prediction. For example, one might have a weather prediction system at Williamsburg, Virginia. If you were to predict that every day was going to be fair, this probably would be a correct prediction 90 or 95% of the time using a very good prediction system. The difficulty with such predictions is that, when there is unusual weather such as rain, one would have to separate the predictions associated with fair weather and predictions associated with rain. Well here I think there is a similar situation, in that over a wide range of situations, no matter what you do, you are going to have failure. And as you predict failure, this may be part of your prediction system but also may be associated with the phenomena under study.

Dr. Hall: Is there any way that you could correct for that, if you know the expected response rate empirically for a particular drug or a particular tumor?

Dr. Zelen: I cannot make a general comment.

Dr. Hall: Indirectly, are you not getting at that by the use of mouse tumors, like Ehrlich ascites, on which many drugs act? In this tumor, the predicting systems have shown that you do pick up a lot of good drugs, whereas, in human tumors, only about 10% of the drugs show activity. So in a very crude way you are correlating with what you know is the case.

I was particularly interested in Dr. DiPaolo's talk and the chart he had on his 89 patients. His negative prediction was 42%, positive prediction 35%; and confirmation for the negatives always correct. Dr. DiPaolo had predicted 47 positives, only 12 of which were correct, *i.e.*, one-quarter. About one-quarter is the best response rate you can expect in treating solid tumors; so it may be that this is just a statement of reality and not a prediction at all.

Dr. DiPaolo: That is possible. We compared Ehrlich ascites, which we grow in culture, with spontaneous mammary gland tumors of mice which are supposedly in most cases not controlled by hormones. We found the same sort of thing that we found with the human material; *i.e.*, the Ehrlich responded as we predicted, while spontaneous tumors responded in the way that human material was responding.

Dr. Wolberg: There may be another factor here. Some of these tumors that one predicts as being sensitive from *in vitro* tests in fact are not. Other factors, such as drug distribution, kinetics, and biochemical sensitivity, lead me to anticipate at least some failure in this situation.

Dr. Bertino: The data with asparaginase show the problems with *in vitro* test prediction. Clearly almost all leukemias will be sensitive, and in almost all lymphatic leukemias the white blood cell count (WBC) will go down. It is a question again of defining what we mean by sensitivity. If we mean decrease in the WBC, the test predicts sensitivity. As Dr. Prager mentioned, the asparagine synthetase turns on in most of the cell types. If the acute lymphatic leukemia is killed before it can turn on, that is a difference rather than a failure of the *in vitro* test.

Participant: This bears on the question that Dr. Hall raised earlier: If we have a pathway in which we have an enzyme which is operating at a very low level of activity or very inefficiently, is that a good place to try to block in order to try to kill the tumor? Does that become a critical enzymatic step? We may have enzymes that are inducible or be repressible, or those that may change the inhibitory level of certain metabolites within the cell when the drug is actually administered. So, simply measuring an enzyme activity before the therapeutic treatment is, I am afraid, going to have a very limited predictive potential. It is only when we see what happens after the therapy that we might be able to cope better with the problem of whether we ought to get a response or whether there is a growing resistance to the therapeutic agent.

Dr. Kessel: Two thoughts occurred to me as I was listening to these discussions. One is that, in many solid tumors, there are a few cells with abnormal karyotypes which seem to be propagated along with the solid tumors. This is despite the fact that they have vastly different growth rates than cells with normal karyotypes, and one would think they would be wiped out. My thought was that these cells were perhaps functioning as "feeder" cells. If there are, indeed, tight junctions in these cells to which asparaginase, for example, has been fed, it probably would be unlikely that you could ever get at this particular type of phenomena with asparaginase, which is mostly an extracellular enzyme. Is this right, that you would not expect asparaginase to enter the cell and destroy it very easily?

Dr. Prager: There is no reason to think it does enter any of the tumor cells.

Dr. Kessel: Another thought occurring to me was that some of these predictor tests will tell a fast-growing from a slow-growing tumor so that you might get a response from fast-growing tumors because they are in general more drug responsive than slow-growing ones. If a certain type of test is capable of making that kind of distinction, it might make a prediction simply on the basis of fast- or slow-growing cells.

Dr. Cline: Of course, when one is looking at actinomycin D inhibition of uridine incorporation into RNA, one can use a cell line which is known to be resistant to actinomycin D, and actinomycin D *in vivo* does not inhibit uridine incorporation of RNA. But, if the cells are simply suspended, this inhibition is seen; but, if the cells are allowed to begin growing in culture, then the inhibition is not observed. So, all I can say is that with uridine incorporation into RNA this effect is not seen, but, on the other hand, the agar test doesn't involve that; it is another enzyme.

Dr. Heidelberger: Dr. DiPaolo has described what seems to be an extremely simple test, and he also described quite a correlation, particularly when negative. He did this when he was at Roswell Park. Was this test used very actively at Roswell Park after you left [Dr. DiPaolo], because it seems to me to have most exciting possibilities?

Dr. DiPaolo: It was to have continued after I left; in fact the grant was turned over to someone else. As to its current status, I have been asked to come back and write a paper, but that doesn't mean that it is still going on. When I think of carcinogenesis, I have in mind three points: 1) the target cell and the insult, 2) the nontarget cell and the insult (which, as you know, may be involved in detoxifying the chemical insult), and 3) the host influence. This sort of test, but not this test specifically, is one that should be explored exhaustively to determine whether it is any good. One of the main factors is the difficulty in getting the piece of tissue from the pathologist.

Participant: Dr. Prager, did you do the test on patients who were sensitive and became resistant to asparaginase; did you repeat the test then, or in the case of a sensitive line do they ever become resistant?

Dr. Prager: Certainly with the mouse tumors the correlation has been excellent. In the clinical situation with respect to the valine uptake, I have to report other people's data rather than my own. There have been some indications that the test results showed increasing resistance, but I cannot tell you what fraction of the time the correlation was good.

Participant: One should at least be aware of the fact that, if one uses bacterial asparaginase, like the *Escherichia coli*, this also destroys glutamine in the tissue culture medium. And you might get what appears to be a positive test result because of glutamine depletion. In the most highly purified preparations of *E. coli* asparaginase I have looked at, the hydrolytic activity against glutamine is about 2.5–3% that against asparagine. Over many hours that could become appreciable.

Dr. Sokal: One can look at tests in various ways. Our approach here is obviously empirical. Someone devises a test, and we hope it is simple and readily usable. It has such and such success in predicting failures and such and such success in predicting responses. To the statistician, a test that is 88% accurate in predicting failure and 89% accurate in predicting successes would be considered pretty good. To the clinician, this might not be as useful as a test that was 100% accurate for either response or resistance, even if it was only 20% accurate for the other. A lot depends on the use to which you put the test. To a physician, who has a very seriously ill patient with practically no bone marrow left and is near death, he figures there is one more chance. Here is a list of four drugs. It is useful for the physician to know that two of the four drugs should not be tried, and that he has a 50% possibility of success with the drug he uses. What I am saying is that we should not diminish the importance of the kind of test Dr. DiPaolo, Dr. Knock, and Dr. Kondo have been describing because it fails on the positive side. If it identifies successfully 100% the agent that will *not* be effective, then the test is very useful to the clinician because it narrows the range of possibilities and improves his one last chance of saving the patient's life. So, I do not care if the test predicts that the drug ought to work and the patient does not respond, so long as the test will positively identify the drugs I should not use. If it is 100% correct on that, then the test is useful.

Dr. Bertino: In resistance to asparaginase, two groups have shown that those patients who are sensitive will become resistant and the asparagine synthetase goes up. Or if an antibody limits use of the drug because the patient has an allergic reaction, this does not necessarily correlate with resistance *in vitro*.

Participant: An antibody can also act as an inhibitor of the enzyme. This has been demonstrated.

Dr. Zelen: Let us suppose the agent is effective in the clinical situation. Dr. DiPaolo correctly predicted that it would be effective in 12 of 12 times. The chances are close to 1 that, if this is a representative sample, they will make a correct prediction. On the other hand, suppose the agent is ineffective. Then the probability, with Dr. DiPaolo's results, is 42 of 77, a little less than one-half. If one was to just keep using this to evaluate the consistency by the probability of making the correct decisions, then you have to weigh them by the proportion of clinically effective agents to ineffective agents. And if you have a high proportion of ineffective agents, then what is going to happen is that the probability of correct results will be closer to one-half than to 100%. There is more to this than just looking at numbers—one has to investigate where these numbers come from.

Participant: I want to answer the point about the antibody to asparaginase. This is a problem clinically but, as far as resistance is concerned, it is not predictable by the test described. In the patients who were about to develop an antibody reaction, it has been recognized that there was an abrupt drop in the asparaginase level in the serum usually just before, or at the time of, the allergic reaction. In the patients who developed resistance without any over-allergic reaction, this fall in asparaginase could not be seen.

Dr. Nickson: A book entitled *Pygmalion in the Classroom* reports the experience in Los Angeles in a depressed area where teachers were told the "natural" IQ's of the children when, in fact, the IQ's were assigned by random numbers.` A very astonishing thing occurred: Teachers behaved toward the kids as if they innately had the IQ number assigned to them and the children responded as though they had these IQ's. In other words, there was a very strong correlation between learning habits and attitudes towards these children.

I say this because I feel that one thing we really have not touched upon very much in this Conference is how to make our observations maximally relevant to the real work. We are dealing with people and our attitudes towards them—we are not dealing with "cancer." If we think we have determined a predictor and then go to patients with an attitude agreeing with the prediction, I think we have to factor out the likelihood that we are predetermining the response to the agents. We have been talking about predictors for cancer, but that is not really what we are talking about or should be talking about. We are talking about people who have cancer and who are given various forms of treatment. I believe it is terribly important to be as careful as we can that, when we have a predictor under test, we set it aside and not have that information fed into the attitudes of the physicians who are making judgments in respect to what is happening to the patients. If we do not do this, there is a very serious danger of establishing a feedback which disturbs the evaluation of the predictor.

PANEL IX

CLINICOEPIDEMIOLOGIC FACTORS

Scientific Defects in the Staging of Cancer[1, 2, 3, 4]

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FOR ALMOST a century, doctors have regarded cancers as neoplastic growths that spread, destroy, and kill. In this concept, cancer is recognized as a biologic force that creates anatomic lesions and functional impairments in an affected host. Because the components of that biologic force have not been fully analyzed, however, many scientific defects now exist in our study of cancer. We have classified and evaluated a cancer's anatomic lesions, but we have not given equal attention to its functional impairments or to other important features of the state of the affected host.

Let us first consider the way a simple physical force is classified in the domain of physics. The force is called a vector, and its components are cited as a group of coordinates for such features as location, magnitude, direction, and time (or velocity). Each of these components is recognized as a distinct element of the vector, and each is given a specific classification.

Now let us consider the way that a much more complex vector—the intricate biologic force of a cancer—is classified in the domain of medicine. We use a group of categories called a "staging system" in which we list coordinates for location (or primary site of the cancer), for magnitude (or size of the cancer), for direction (or topographic spread of the cancer), and for histologic type (or microscopic appearance of the cancer). All these coordinates, however, deal only with the anatomic morphology of the cancer. The existing staging system does not provide coordinates for such important features as the rate of growth of the cancer, or its functional effects, or the associated ailments and diseases that may coexist in the affected patient. For example, we often talk about "early" detection of cancer, but we do not assess any evidence of time to substantiate the chronometric implications of the word "early." We often say that a cancer has been detected "early" if it is small and has not spread, but the actual evidence that we observe consists of data about size and topographic extensiveness, not time. The evidence is based on anatomy rather than chronometry.

COORDINATES OF THE CANCER VECTOR

Suppose we wanted to construct a vector with coordinates for classifying the biology of a cancer. What coordinates do we want to include? According to what we have just discussed, we want to provide for anatomic features, for functional features, and for the environmental aspects of the affected host.

Anatomic Features

Topographic extensiveness is an obvious anatomic feature, and it is the mainstay of current systems of anatomic staging. Another important anatomic property is the external margination that would permit surgical resection of a cancer. For example, a localized cancer of the lung might not be resectable because it is situated at the tracheal carina, without a surrounding margin of normal tissue that would allow its safe removal. We also might want to know anatomically about the size of the cancer, although total size is notoriously difficult to measure without removal of the cancer and, even after removal, is almost impossible to assess if the cancer has spread diffusely. Another standard anatomic feature of current staging is the microscopic type of the cancer, despite the occasional unreliability of these identifications because of frequent changes in histologic criteria and un-

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[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

[3] Supported in part by Public Health Service grant HS 00408 from the National Center for Health Services, Research, and Development.

[4] Send *requests for reprints* to VA Hospital, West Haven, Conn.

standardized observer variability among pathologists *(1)*.

Functional Features

Among the functional coordinates we might like to know about are the tumor's iatrotropic effects *(2)*. Did the patient seek medical attention because of symptoms due to cancer; or did he come to a doctor because of symptoms due to some other disease; or was the patient asymptomatic, with his cancer detected in some routine screening examination? If the patient was symptomatic, what kind of symptoms did he have? Were they primary symptoms—such as hemoptysis in lung cancer or bleeding in rectal cancer—that are attributable to the tumor at its primary site or to surrounding inflammation? Were they systemic symptoms—such as anorexia, fatigue, and weight loss—that arise holistically in the body without implying an anatomic spread of the cancer? Were they metastatic symptoms—such as bone pain and neurologic manifestations—that imply, by their very existence, an anatomic spread of the cancer? Did the patient have primary symptoms alone, or various combinations of primary, systemic, and metastatic symptoms *(2)?*

What had been the sequence of those symptoms? Had the patient been coughing blood and did he then start to become anorectic and fatigued, or did he first become anorectically fatigued and then start coughing blood? What had been the chronometric duration of the overt manifestations of the cancer? How long had the patient been having his symptoms, or how long had a lesion been noted to be previously present on X rays or physical examinations? Finally, what about the functional effects that might be called "para-morbidity"? These effects are demonstrated by various laboratory tests, indicating such features as anemia, hypercalcemia, and hyperglobulinemia.

Environmental Aspects of the Affected Host

The human host who serves as the "environment" for the cancer's growth must be classified for *comorbidity,* which refers to the major coexisting ailments that might be present in addition to the cancer and that might have major effects on the outcome of the clinical course *(3)*. We must also consider such demographic features of the host as age, race, sex, and occupation.

STATISTICAL PROBLEMS

We do not know in advance which of these different coordinates are likely to be important indexes of the biologic behavior of a cancer. We cannot be sure of their relative importance until we have examined them, not with anecdotal recollections and judgmental evaluations, but with specific categories of identification, formal systems of classification, and quantitative enumeration. But these examinations have generally not been done. The topics of statistical analysis have been the topographic extensiveness designated by anatomic staging, the histologic categories, and occasional demographic features such as age, race, and sex. All the functional features just described and the diverse effects of comorbidity have been omitted from the statistical analyses because these coordinates have not been classified. A distinctive system of taxonomy has not been developed that would identify them, give them categories, and permit their formal analyses.

And yet, if these functional and comorbid features are important, they will demarcate major prognostic gradients within a group of patients who appear deceptively similar because they have the same anatomic stage and demographic attributes. These gradients will mean that our anatomic stages and demographic groups have fallaciously been regarded as homogeneous—that these stages and groups are really heterogeneous, denoting major disparities in biologic behavior of the cancer. But these stages and groups would have been spuriously regarded as suitably well defined for our studies of neoplastic pathogenesis and therapy.

CLINICO-STATISTICAL EVIDENCE

During the past few years, my colleagues and I have been exploring these neglected coordinates of the cancer vector. We have developed systematic, thorough methods for obtaining the data, and we have created systems of taxonomy for classifying the information *(4–6)*. The rest of this discussion is concerned with specific evidence of the prognostic gradients and biologic indexes of cancer that can be discerned from such features as iatrotropy, symptom clusters, chronometry, and comorbidity.

The prognostic gradients of conventional anatomic staging are shown in table 1, whch contains the 5-year survival rates, regardless of treatment, for 678 patients, who represent the entire population with primary lung cancer seen at the Yale-New Haven and West Haven Veterans Administration Hospitals during 1953–59. The overall 5-year result was 7% survival. In the localized Stage I, however, the survival was 16%; in the regionally spread Stage II, 4%; and in the distantly disseminated Stage III, 1%. This is the customary kind of gradient noted in anatomic staging, and the results here are quite similar to those noted in other studies of the same problem *(7–9)*.

Table 1.—Five-year survival rate in lung cancer (all types of therapy): Anatomic stage

Anatomic stage			
I—localized	II—regional	III—distant	Total
35/218 (16%)	8/180 (4%)	2/280 (1%)	45/678 (7%)

The data in table 2 demonstrate the prognostic gradient demarcated by symptomatic stages, based on a taxonomy of symptoms defined elsewhere *(10, 11)*. In the same 678 patients just cited, the classification into 3 symptomatic stages showed that the best survival rate, 17%, was in the *indolent* Stage I; next best, 7%, in the *obtrusive* Stage II; and worst, 0%, in the *deleterious* Stage III.

Table 2.—Five-year survival rate in lung cancer (all types of therapy): Symptomatic stage

Symptomatic stage	No. of survivors/Total No. patients
I: indolent	21/125 (17%)
II: obtrusive	24/334 (7%)
III: deleterious	0/219 (0%)
Total....................	45/678 (7%)

The relatonship of symptomatic and anatomic stages for these patients is shown in table 3. For each anatomic stage, a distinct prognostic gradient was present in the vertical descent from symptomatic Stage I to symptomatic Stage III: The rates were 24, 15, and 0% in anatomic Stage I; 10, 5, and 0% in anatomic Stage II; and 4, 1, and 0% in anatomic Stage III. Similarly, within each symptomatic stage, a distinct prognostic gradient was present in the horizontal transition from anatomic Stage I to anatomic Stage III.

Table 3.—Five-year survival rate in lung cancer (all types of therapy)

Clinical (symptomatic) stage	Anatomic stage			
	I	II	III	Total
I	17/71 (24%)	3/31 (10%)	1/23 (4%)	21/125 (17%)
II	18/118 (15%)	5/100 (5%)	1/116 (1%)	24/334 (7%)
III	0/29 (0%)	0/49 (0%)	0/141 (0%)	0/219 (0%)
Total........	35/218 (16%)	8/180 (4%)	2/280 (1%)	45/678 (7%)

These interactive gradients are present in rectal cancer as well as in lung cancer. Table 4 contains the data for 221 patients whose rectal cancer was resected at the Yale-New Haven and West Haven Veterans Administration Hospitals during 1953–61. The 3 anatomic stages showed, in total, a 5-year survival gradient of 57, 26, and 6%; the 3 symptomatic stages showed, in total, a gradient of 52, 33, and 0%; and for the 9 clinico-anatomic categories, there were prognostic gradients vertically within each anatomic stage and horizontally within each symptomatic stage.

Table 4.—Five-year survival rate after resection of rectal cancer

Clinical (symptomatic) stage	Anatomic stage			
	I	II	III	Total
I	36/50 (72%)	6/15 (40%)	1/18 (6%)	43/83 (52%)
II	34/70 (49%)	6/31 (19%)	2/28 (7%)	42/129 (33%)
III	0/2 (0%)	—	0/7 (0%)	0/9 (0%)
Total........	70/122 (57%)	12/46 (26%)	3/53 (6%)	85/221 (38%)

BIOLOGIC IMPLICATIONS

These gradients within gradients have several important biologic implications. They indicate that we must classify both form and function in cancer, rather than anatomic form alone or symptomatic function alone. The gradients also indicate the reasons for some of our current controversies about therapy. If two surgeons, two radiotherapists, or two chemotherapists claim to be treating anatomic Stage I or anatomic Stage II, they will get major differences in results according to whether the patients are in clinical Stages I or III. With the symptomatic distinctions currently neglected, major differences after treatment may be fallaciously attributed to the treatment, instead of to the initial prognostic differences of the patients.

This problem cannot be solved merely with randomized allocations of treatment while the prognostic distinctions remain ignored. Randomization may eliminate bias in allocation of treatment and may allow statistical parametric estimations, but it can provide neither the scientific precision nor the clinical meaning that would be desired in therapeutic trials. The kind of biologic specificity wanted by clinical scientists would require a careful identification of distinctive different phenomena, rather than a "promiscuous" randomization that obscures their differences by lumping them all together imprecisely.

Finally, let us consider the work of a pathogenetically oriented biochemist who is studying the enzyme content of a collection of adenocarcinomas. He may find major variations in the range of an enzyme and may conclude that the enzyme had no particular meaning or significance. Nevertheless, if he had noted what kind of functional cancer he dealt with, he might have found that the high enzyme values were in symptomatic Stage I of cancer but not in symptomatic Stages II or III. In this way, many promising biochemical and other clues to the pathogenesis of cancer may be overlooked or discarded because a suitable correlation has been neglected.

APPRAISALS OF SURGERY

Since the 9 clinico-anatomic categories we have been considerng create too many groups for many statistical purposes, we would like to compress them while preserving the virtues of both symptomatic and anatomic stages. This compression can be achieved as shown elsewhere *(11)*, if clinico-anatomic Stage A is assigned to patients who are clinically and anatomically in Stage I. Stage B then consists of patients who are clinically Stage I and anatomically Stage II (or vice versa); Stage C consists of patients who are clinically and anatomically in Stage II, and Stage D consists of patients with either anatomic or symptomatic evidence of metastasis.

During the perennial debates about the merits of simple versus radical surgery for cancer, any apparent statistical advantage for either mode of treatment has usually been countered with the complaint that the compared patients were not really comparable. With a reasonable comparability established by the new clinico-anatomic categories, the results of simple versus radical surgery in rectal cancer are shown in table 5. The data of table 5 were confined to patients whose rectal cancer was located at least 8 cm above the anus, so that the surgeon could elect to do either the radical abdomino-perineal resection that removes the anus, or a simpler procedure with anastomosis or other types of resection that would preserve the anus. The overall results of 5-year survival were essentially the same in both groups: 40 and 41%. But the rates were also essentially the same in each of the 4 clinico-anatomic categories: 67 versus 63% in Stage A; 56 versus 62% in B; 25 versus 19% in C; and 7% for both of the D groups.

TABLE 5.—Five-year survival rates in simple-versus radical surgery in rectal cancer (tumors located 8 cm or higher above anus)

Clinico-anatomic stage	Anastomotic or other resection preserving anus	Abdomino-perineal resection
A	8/12 (67%)	10/16 (63%)
B	9/16 (56%)	15/24 (62%)
C	1/4 (25%)	3/16 (19%)
D	1/15 (7%)	1/14 (7%)
Total............	19/47 (40%)	29/70 (41%)

OTHER ASPECTS OF THERAPY

For the consideration of methods of treatment other than surgery, the survival period in the remaining discussion here was reduced from 5 years to 6 months. As shown in table 6, the 678 patients with lung cancer had a 6-month survival rate of 42%. The rates were 73% for the patients who had a surgical thoracotomy and whose tumor was excised, 58% for the patients who had thoracotomy without excision of the cancer, and 27% for the patients who had no surgery. Because the difference between the 58% and the 27% is highly

Table 6.—Six-month survival rates in lung cancer

Mode of treatment	Survival rate
Surgery with excision	141/192 (73%)
Surgery with no excision	40/69 (58%)
No surgery	107/417 (27%)
Total	288/678 (42%)

significant, a clinically naive observer might draw a major conclusion from the data. "Aha," he might say, "If a patient is inoperable, we ought to open and close his chest anyhow because it seems to be so good for him." Unfortunately, this type of naiveté sometimes appears in statistical analyses that attribute all the post-therapeutic results to the effects of treatment, and that ignore the initial differences in the state of the patients. What these data indicate, of course, is the surgeon's ability to select the favorable cases for surgery, so that even when the cancer cannot be resected, the patients chosen for surgery have better outcomes than those who were deemed inoperable. Some of the surgeon's selection techniques are shown in table 7, which quantifies the way that surgeons pick their "operable" cases mainly from the more favorable A, B, and C stages, leaving the D groups behind for the radiotherapist and chemotherapist. Another aspect of surgical selection is shown in table 8, which indicates the low rates of comorbidity in the "operable" cases, as compared to the much greater occurrence of major comorbid diseases in the "inoperable" groups. Thus, the surgeon preselects the best clinico-anatomic stages and the "healthiest" patients without comorbidity—but he then often proceeds to compare the therapeutic virtues of surgery against the results of radiotherapy or chemotherapy for the inoperable "dregs" that he leaves behind.

Table 7.—Distribution of clinico-anatomic stages according to mode of therapy in lung cancer

Clinico-anatomic stage	Surgery (with or without excision)	No surgery	Total
A	60 (23%)	11 (3%)	71 (10%)
B	103 (39%)	46 (11%)	149 (22%)
C	54 (21%)	46 (11%)	100 (15%)
D	44 (17%)	314 (75%)	358 (53%)
Total	261 (100%)	417 (100%)	678 (100%)

Table 8.—Occurrence of significant comorbidity in population of lung cancer patients treated with and without surgery

Clinico-anatomic stage	Rate of comorbidity	
	Surgical	Nonsurgical
A	6/60 (10%)	5/11 (45%)
B	3/103 (3%)	22/46 (48%)
C	3/54 (6%)	14/46 (30%)
D	2/44 (5%)	80/314 (25%)
Total	14/261 (5%)	121/417 (29%)

Another important feature of comorbidity is its effects in the inoperable patients who are often studied in trials of chemotherapy. Table 9 indicates the results for the 417 patients whose lung cancers were inoperable. Although the total 6-month survival rate was 26%, it was 40% for the patients in Stages A, B, or C and 21% for the majority of inoperable patients, who were in Stage D. For the patients with major comorbid ailments, the total survival rate was 17%; but it was 29% for patients without comorbid ailments. In the commonly occurring Stage D, the survival of patients who were comorbid was 8%, whereas it was 26% in those who were not comorbid. By contrast, the survival rate was as high as 43% in the noncomorbid patients of Stages A, B, or C. Bearing these distinctions in mind, we can ask a blunt scientific question: When people study new chemotherapeutic agents and report good or bad results in "inoperable" patients, without further specification, what kind of patients are they studying?

CONCLUSION

The biologic behavior of a cancer requires analysis of more than just its morphologic appear-

Table 9.—Six-month survival rates for "inoperable" patients with lung cancer

Clinico-anatomic stage	With comorbidity	Without comorbidity	Total
A, B, or C	14/41 (34%)	27/62 (43%)	41/103 (40%)
D	6/80 (8%)	60/234 (26%)	66/314 (21%)
Total.........	20/121 (17%)	87/296 (29%)	107/417 (26%)

ance. In addition, we must classify and analyze not only its functional clinical effects, but also the comorbid ailments that may be present in the affected patient. The complexity of man increases the difficulty of studying human disease, but also enables a diseased man to talk. What he tells us about his symptoms and other clinical phenomena can give us major clues to the scientific biology of cancer, if we will listen to him carefully, classify what we learn, and analyze it appropriately.

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Data Analysis Methods for Inferring the Natural History of Chronic Diseases[1, 2, 3]

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THE STUDY of the natural history of a chronic disease is important for evaluating the benefit of therapy, for making useful prognosis about survival after diagnosis and treatment, and to throw light on the possibly increased benefit of therapy if the disease is diagnosed earlier. Of course, it is difficult to study the natural history of a disease directly, because once the disease is diagnosed and treated, its eventual course may not be the same as if the disease had not been treated. For example, with respect to breast cancer, the primary tumor is removed after diagnosis and cannot be studied as part of the disease process. One may attempt to study the natural history of a disease by studying data from patients treated in an earlier period when the method of treatment was not effective. However, studies of this kind are handicapped because, usually, only fragmentary information is available about the course of the disease in patients treated long ago.

Among the crucial questions one hopes to answer by studying the natural history of a neoplastic disease are:

a) What proportion of the diseased population is "cured" by a particular type of therapy?

b) How many years of life are lost due to an individual having a chronic disease?

c) What is the relationship between earlier detection of the disease and increased benefit from therapy?

These questions are all related to each other and in turn depend on whether a disease is a progressive one; *i.e.,* a disease that progresses and prognosis becomes relatively poorer as time goes on and the disease is not detected.

PRELIMINARY CONSIDERATIONS

The years of life lost due to having cancer is related to whether the disease is localized or metastatic when treated. With respect to an individual, the time course of the disease may be divided into a *local* phase and a *metastatic* phase. If the disease is found and treated while it is localized, the individual will be cured. On the other hand, if diagnosed and treated in the metastatic phase, the treatment is only palliative and the life of the individual will be shortened. It will generally make little difference whether the metastatic form of the disease is found early or late in the metastatic phase. In such cases the occurrence of death due to disease is predetermined. Text-figures 1a and 1b show the relation between probability of a cure (probability disease is localized when diagnosed) and years lost due to having the disease as a function of time of detection for an individual.

However, the characteristics of the course of the disease for a population of patients require three phases: *local phase, local–metastatic phase,* and a *metastatic phase.* Consider a population of patients all having the disease for the same length of time. This group will be considered to be in the *local* phase if, at that time, they are all treated and cured. The population of cases will be considered to be in the *local–metastatic* phase if the group consists of a mixture of individuals with both local and metastatic disease. Finally, if the entire group consists of individuals in the metastatic phase, the phase of the disease at that time will be regarded as being in the *metastatic phase.* Text-figures 2a and 2b describe the general relation between probability of cure and years lost due to having the disease as a function of time with disease.

Suppose a disease is a progressive one with the characteristic that at time of inception it is local, but, as time goes on and the disease is not diagnosed and treated, it will eventually metastasize. Then one would expect a graph plotting years lost due to having the disease versus time with disease as resembling text-figure 2b for a population of patients. Of course, in many cancers, it may be difficult to diagnose patients in the local phase, and one may only have treatment in the local–metastatic and metastatic phases. In some instances,

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[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

[3] Supported by Public Health Service grant CA 10810 from the National Cancer Institute.

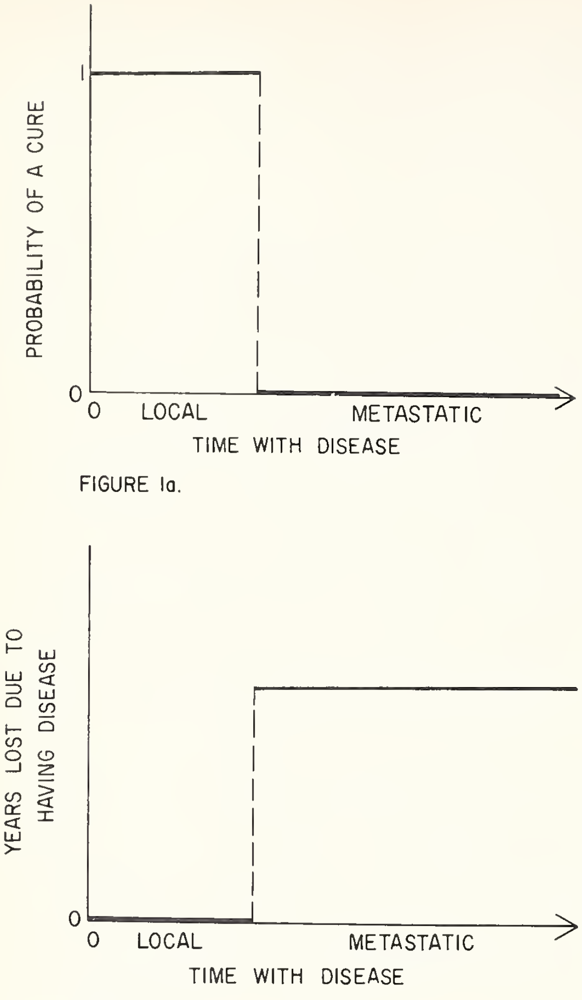


FIGURE Ia.

FIGURE Ib.

Text-figure 1.—*a)* Probability of cure versus time with disease for an individual patient. *b)* Years lost versus time with disease for an individual patient.

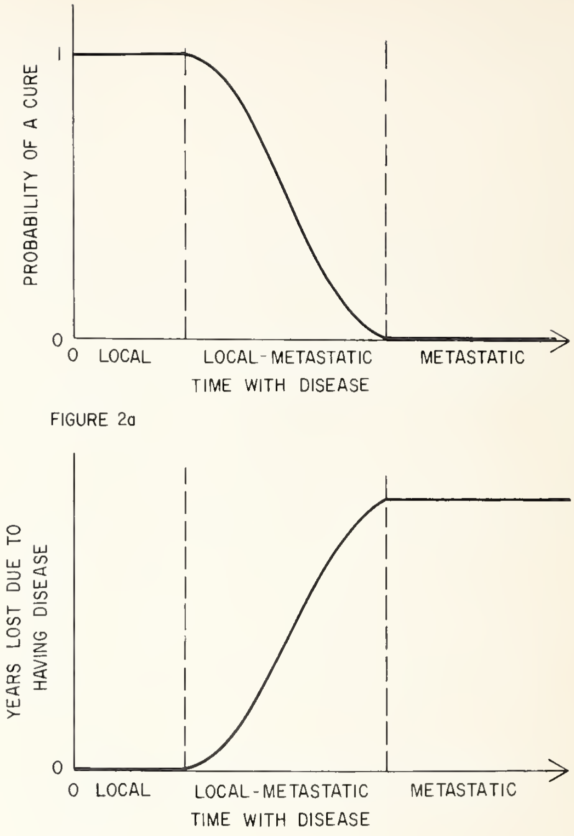


FIGURE 2a

FIGURE 2b.

Text-figure 2.—*a)* Probability of cure versus time with disease for a population of patients. *b)* Years lost due to having disease versus time with disease for a population of patients.

diagnosis and treatment occur only in the metastatic phase. In this case, the treatment is only palliative.

In a practical situation, one may be able to estimate the years lost due to having the disease. This may be done by taking as a measure of the years of life lost due to having the disease the difference between the survival of the normal population and the survival of persons with disease. More explicitly, consider a population of cases, who have all received the same therapy for a particular cancer site. Divide this group of patients into several subgroups or stages so that cases in the same subgroup have more nearly the same disease status at time of treatment compared to cases in different subgroups. Let the median age at treatment for a particular subgroup be z. Then the *Deficit Survival* of this subgroup of patients is defined by

$$(1)\quad \begin{array}{c}\text{Deficit}\\ \text{survival}\end{array} = \left\{\begin{array}{l}\text{median survival for}\\ \text{those in the normal}\\ \text{population who have}\\ \text{lived at least } z \text{ years}\end{array}\right\} - \left\{\begin{array}{l}\text{median survival of sub-}\\ \text{group of cases}\end{array}\right\}$$

The deficit survival is easily estimated for almost any series of cases and clearly reflects the years of life lost due to having the disease. Its behavior corresponds to the curve in text-figure 2b.

Thus, for a practical situation, one may estimate the ordinates of the curve in text-figure 2b, but it does not seem possible to estimate how long the group of patients has had the disease (abscissa). Nevertheless, knowing the ordinate may enable one to classify the subgroup of patients into one of the three disease phases: local, local–metastatic, or metastatic. Clearly, if the deficit survival associated with a group is zero (except for random fluctuations), the individuals in this group can be regarded as being in the local phase when treated. The cases in these subgroups having a zero deficit survival may be regarded as "cures." If there are several subgroups exhibiting a large deficit survival which is nearly constant for all groups, we would classify these subgroups as being in the metastatic phase at time of treatment. Finally, if one has several subgroups which can be ordered with respect to disease severity at time of treatment and the deficit survivals show an increasing trend, then these subgroups are identified as being in the local–metastatic phase.

IDEALIZED PROGRESSIVE DISEASE MODELS

A Simple Three-State Model

To illustrate ideas, consider an "idealized neoplastic disease" such that at time of diagnosis and treatment a patient's disease status can be characterized as being in one of three possible disease states. We shall designate these states by S_1, S_2, and S_3. The disease states are chosen such that, if the disease is a progressive disease, *i.e.*, the disease advances as time goes on and is not detected, the path of the disease is $S_1 \rightarrow S_2 \rightarrow S_3$. This sequence defines the natural history of the disease. If one knows how long it takes for an individual to go from one state to another, we will have the natural time history of the disease.

On the other hand, if the disease is not progressive, there are five other possible paths which the disease may take:

$S_1 \rightarrow S_3 \rightarrow S_2$
$S_2 \rightarrow S_3 \rightarrow S_1$
$S_2 \rightarrow S_1 \rightarrow S_3$
$S_3 \rightarrow S_1 \rightarrow S_2$
$S_3 \rightarrow S_2 \rightarrow S_1$

We wish to determine the characteristics of the observed data if the disease is indeed progressive.

Assuming the disease is progressive ($S_1 \rightarrow S \rightarrow_2 S_3$), we would expect that: 1) *The survival of an individual treated in an earlier state will tend to be greater than the survival associated with a state to which the patient may eventually enter at a later time.* 2) *The age at diagnosis associated with a state will tend to be younger compared to the age of diagnosis of states reached at a later time.* 3) *The deficit survival of an individual treated in an earlier state will tend to be smaller or remain the same compared to the deficit survival associated with a state to which the patient may eventually enter at a later time.*

If the observed data fail to satisfy the above three conditions, this implies that the disease is not a progressive disease as characterized by the three states. We shall refer to the above three conditions as the *Progressive Disease Data Postulates.*

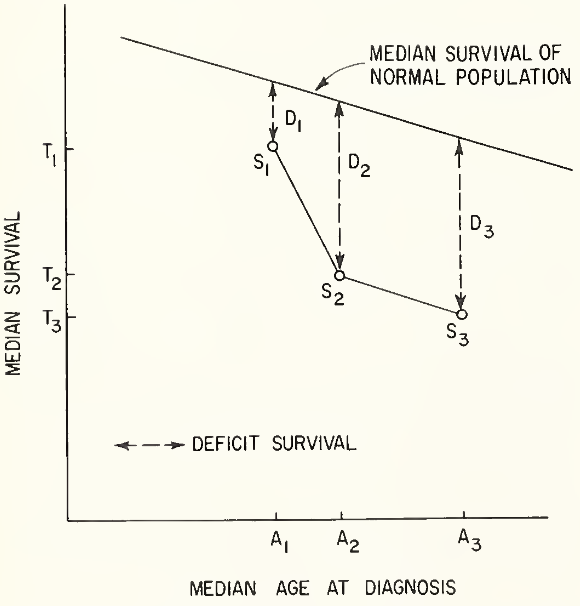
To be able to operationally check these postulates we will use the median survival time as a measure of survival and the median age of diagnosis to summarize the location of the age distribution. Hence, the operational uses of postulates 1 and 2 are: 1) median survival of an earlier state is greater than at later states; and 2) median age of diagnosis is younger at earlier states.

Implicit in Postulate 1 is that treatment in an earlier state of the disease will tend to result in better survival. Postulate 2 merely states that people become older as time goes on. However, age is a key piece of information which enables one to put a time scale on the progressive disease path. Note that Postulate 3 does not allow the deficit survival to become smaller as the disease progresses. This would imply that delay in diagnosis benefits the patient. Further, if any of the disease states has zero deficit survival, this means that the state is a "curative state" and the disease is treated in the local phase. That is, individuals diagnosed and treated in that state have the same median expectancy of life as individuals in the normal population. On the other hand, if the deficit survival increases with advancing disease state, we would conclude that delay in detection and treatment results in increased loss of life. This would place the states in the local–metastatic phase of the disease. One could then calculate the expected loss in life for each year of delay. Finally, the states would be judged to be in the *metastatic phase* of the disease if the deficit survival is constant for all three states. Clearly, the pattern of the deficit survival information is important for interpreting the benefit of therapy and the importance of earlier detection of the disease.

The relations between the median age, survival, and deficit survival can be most easily exhibited graphically. Suppose we define:

A_i = median age of diagnosis in state S_i
T_i = median survival in state S_i
$N(A_i)$ = median survival in normal population for individuals who have lived at least A_i years
D_i = (deficit survival for state S_i) = $N(A_i) - T_i$.

Thus, for each state S_i we have the information (A_i, T_i, D_i). The age at diagnosis (abscissa) and survival time (ordinate) can be used as coordinates to plot each state. If the disease is progressive, then state S_{i+1} will have older age at diagnosis and shorter survival time than state S_i; *i.e.*, $A_i < A_{i+1}$, $T_i > T_{i+1}$. Therefore, a more advanced disease state will be located southeast of a less advanced disease state. A typical plot for states satisfying the Progressive Disease Postulates is given in text-figure 3. The deficit survival is denoted by the vertical arrows. From this plot, it is clear that *a*) none of the states could be classified as being in the local phase; *b*) the deficit survival of states S_2 and S_3 is the same ($D_2 = D_3$); hence it makes no difference whether the disease is treated in states S_2 or S_3; *c*) the deficit survival associated with S_1 is smaller than survivals for S_2 and S_3; therefore, earlier detection and treatment in state S_1 are beneficial to the patient. An estimate of how long it takes to go from state S_i *to* S_{i+1} is simply the difference in the median age of diagnosis; *i.e.*, $A_{i+1} - A_i$.



Text-figure 3.—Hypothetical plot showing relations between age at diagnosis, median survival, and deficit survival.

Construction of States Based on TNM System

The TNM system of staging has been put forth by the International Union Against Cancer (UICC) as an international uniform staging system for various disease sites. Adapting the breast system TNM staging, we shall consider the primary tumor (T), regional lymph nodes (N), and distant metastases (M) to be made up of two conditions termed favorable and unfavorable as defined below.

Disease variable	Favorable	Unfavorable
T: Primary tumor	Smaller than 2 cm	2 cm or larger
N: Regional lymph nodes	No evidence of nodal involvement	Nodal involvement
M: Distant metastases	No evidence of distant metastases	Evidence of distant metastases

Since we have three disease variables, each at two conditions, the total number of disease states is $2^3 = 8$. These will be designated by combinations of lower case letters involving *t, n, m* where the presence of the letter means favorable in that state, whereas absence of the letter denotes an unfavorable condition. The eight disease states are summarized below:

State	T	N	M	Stage
tnm	F	F	F	I
nm	U	F	F	III
tm	F	U	F	II or III
tn	F	F	U	IV
t	F	U	U	IV
n	U	F	U	IV
m	U	U	F	III
(U)	U	U	U	IV

The state where all disease variables are unfavorable is denoted by *(U)*. The last column indicates the appropriate stage according to the UICC classification.

Evidence of distant metastases will, in almost all cases, be also accompanied by regional nodal involvement. Hence the two states *(tn, n)*, where both regional nodal involvement is favorable and distant metastases are unfavorable, will be neglected. Then the possible paths, if the disease is progressive, are:

(II or III) (IV)
tm *t*
tnm *(U)* (IV)
(I)
nm *m*
(III) (III)

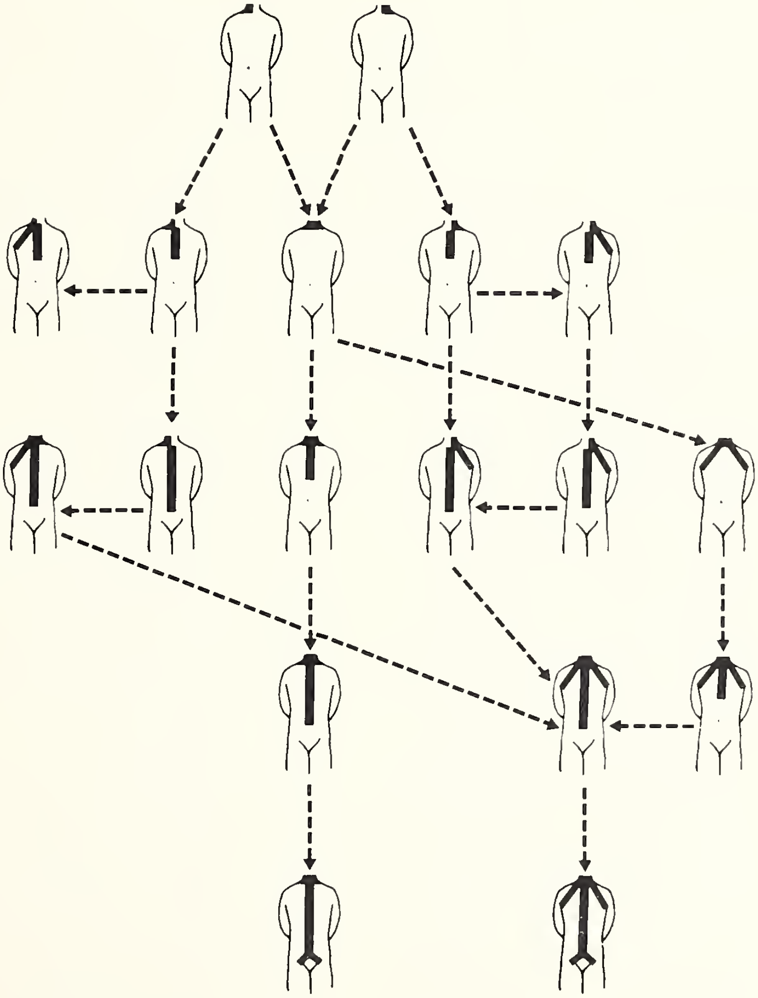
The above pattern only summarizes all possible natural history paths. It would be necessary to have the age at diagnosis and survival data to determine which subset of paths are supported by the data. Note also that states *m* and *(U)* are multiple entry states; *i.e.*, patients enter these states from two different states. In these cases it would be important to search the bivariate distribution of age at diagnosis and survival time (for individuals) to determine if the distribution of the data has more than one mode.

States for Hodgkin's Disease

As an example of another way of constructing disease states, we shall briefly examine Hodgkin's disease. There is increasing evidence that Hodgkin's disease is a progressive disease. Recent advances in diagnosing Hodgkin's disease have led to more accurate location of the extent of involvement of disease. We shall take as the state of the disease the various possibilities for involvement. Text-figure 4 shows the possible paths for involvement of Hodgkin's disease. [These figures represent the most common involvement for untreated cases reported by Rosenberg and Kaplan *(1)*.] It would be of great interest to determine which of the many paths satisfies the Progressive Disease Postulates. These states may further be enlarged by the addition of the histologic classification added on to each disease site.

NATURAL TIME HISTORY OF BREAST CANCER

In this section we discuss the application of the method to retrospective data on breast cancer. The results are reported more fully by Zelen *(2)*.



TEXT-FIGURE 4.—Patterns of possible path for Hodgkin's disease.

Despite the many studies devoted to breast cancer, there is controversy about fundamental aspects of the disease. There is little quantitative evidence of the therapeutic gain with earlier detection of the disease; even the value of radical mastectomy is questioned by several investigators. Furthermore, technical questions exist about the importance of the histology of the primary tumor; is the degree of sinus hyperplasia of the axiliary lymph nodes (sinus histiocytosis) indicative of a host defense mechanism? What is the effect of menopausal status on prognosis? Do younger women have poorer prognosis? The natural history of breast cancer appears to be very complicated, depending on the biological properties of the neoplasm, possible host resistance, and menopausal status of the patient.

The data we shall use are a retrospective series of breast cancer cases diagnosed and treated at the Yale–New Haven Medical Center during 1921–53. The original series, consisting of 1,458 cases, has been discussed by Goldenberg *et al. (3)*. Cutler *et al. (4)*, and Myers *et al. (5)* have discussed these cases in relation to the significance of prognostic factors.

We shall investigate the natural history of breast cancer with disease classification scheme based on: *A*) lymph node involvement, *B*) nuclear grade, *C*) sinus histiocytosis, and *D*) tumor size. It will be convenient to refer to these disease variables by the letters *A, B, C,* and *D*, respectively. Each of these disease variables is taken to be in either a favorable or unfavorable condition at the time of diagnosis and treatment. Since there are four factors, we have $2^4 = 16$ possible disease states. The classification for each disease variable is briefly summarized below:

Disease variable	Favorable	Unfavorable
A. Axillary lymph nodes	Not involved	Involved
B. Nuclear grade	Tumors having some degree of nuclear differentiation	Tumors having minimal nuclear differentiation
C. Sinus histiocytosis	Lymph nodes with marked evidence of histiocytic cells	Lymph nodes with little or no evidence of histiocytic cells, or all available lymph nodes are extensively involved by metastic carcinoma
D. Tumor size	Smaller than 5 cm	5 cm or larger

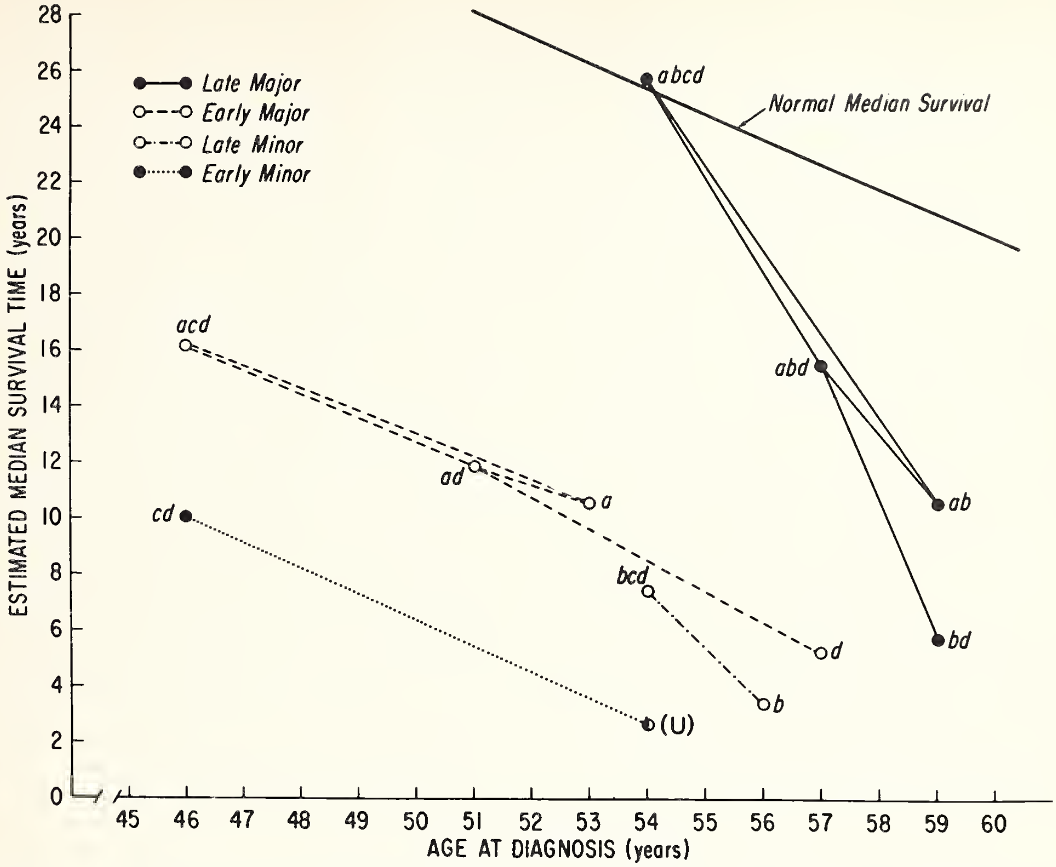
Among the total series of 1,458 cases, there were 467 cases that included full information on all four variables. A number of cases were excluded due to late diagnosis (1954 and later), as were those cases having distant metastases or bilateral cancer. The median survival of these 467 cases was 5.6 years compared to 4.2 years for the entire series. Furthermore, among the 467 cases, all but 4 patients had a radical mastectomy. The remaining 4 had a simple mastectomy.

It will be convenient to use combinations of lower-case letters to denote the disease states. The presence of a letter indicates favorable disease status; the absence of a letter denotes unfavorable status with respect to the disease factor associated with the missing letter. For example, the state *abcd* is the state where all disease variables are favorable. The state *abd* corresponds to unfavorable status for sinus histiocytosis *(C)* and favorable status for the other three disease variables. The state where all four variables are unfavorable will be denoted by *(U)*.

Table 1 summarizes the number of patients, the estimated median survival, and the median age at diagnosis. Text-figure 5 is a plot of the disease states (age at diagnosis vs. survival), omitting those states with fewer than 10 cases. The plot of the states indicates that there are four distinct

TABLE 1.—Median survival time and median age at diagnosis for all disease states

Disease state	Number of patients	Number of breast-cancer-related deaths	Estimated median survival (years)	Median age at diagnosis (years)
abcd	30	6	25.6	54
abc	10	3	16.5	57
acd	15	5	16.1	46
abd	61	20	15.4	57
ad	34	14	11.8	51
ab	21	8	10.5	59
a	23	10	10.5	53
ac	5	2	10.3	58
cd	12	6	10.0	46
bd	67	43	5.7	59
bcd	12	5	7.4	54
bc	7	4	6.7	53
d	48	34	5.2	57
b	55	45	3.4	56
c	5	4	3.4	59
U	62	51	2.6	54



TEXT-FIGURE 5.—Plot of median age at diagnosis versus estimated survival for 12 states. (States with small numbers of patients are omitted.) *Lines joining disease states* refer to transitions for the adjusted principal paths. Presence of *letter* denotes favorable condition of disease variable: *a)* nodal involvement; *b)* nuclear grade; *c)* sinus histiocytosis; and *d)* tumor size.

time histories. The initial disease states of each of these are *abcd, bcd, acd,* and *cd.* These states represent the earliest clinical diagnosed state for each time history.

Note that the median age of diagnosis for both *abcd* and *bcd* is 54 years, whereas the median age of diagnosis for both *acd* and *cd* is 46 years. Thus the time histories beginning with *abcd* or *bcd* are associated with older women who tend to be postmenopausal. We thus conclude that the four time histories generate a distribution of incidence rates having starting points in time which are approximately 8 years apart. Consequently, if one adds the incidence rates associated with the two pairs of time histories, we would expect to find a discontinuity in the total incidence rate when appreciable numbers of patients are diagnosed as having the later established disease. This would show up as a "break" or "dip" in the *total incidence* rate at 54 years of age.

We refer to the time histories beginning with the initial states *abcd* and *bcd* as the *late major* and *late minor* histories, respectively. The *late* refers to the initial state beginning at 54 years of age; *major* and *minor* refer to the relative number of patients in each history. Similarly, the histories beginning with *acd* and *cd* will be referred to as the *early major* and *early minor* histories.

Table 2 summarizes the four paths of the disease as described in text-figure 5. Note that all four initial states are favorable with respect to sinus histiocytosis *(C).* The first change in a disease variable for each path is a change from favorable to unfavorable sinus histiocytosis. Thus this is a biological event which occurs early relative to other biologic events which will subsequently occur. Another interesting observation is that the nuclear grade *(B)* does not change at all with advancing disease. In fact, the distinguishing characteristic of *early* and *late* disease is that favorable nuclear grade is only associated with *late* disease.

TABLE 2.—Summary of principal paths

History	Number of patients	Median age of initial state (years)	Principal path
Late major	179	54	$\begin{tikzcd} abd \arrow[r] & bd \arrow[d] \\ abcd \arrow[u] \arrow[r] & ab \end{tikzcd}$
Early major	122	46	$\begin{tikzcd} & ad \arrow[r] & d \arrow[d] \\ acd \arrow[ur] \arrow[rr] & & a \end{tikzcd}$
Late minor	67	54	$bcd \rightarrow b$
Early minor	74	46	$cd \rightarrow U$

Table 3 groups the four histories and summarizes the median age, median survival, normal median survival, delay in diagnosis from initial state, and the deficit survival. The only disease state having a zero deficit survival is *abcd*. All other groups have a deficit survival significantly different from zero. Hence, the proportion of women in this group of 467 cases is 30/467 = 0.06. This is undoubtedly an overestimate, as the 467 cases analyzed were biased upward relative to the entire series. Inspection of the deficit survival for the *late major* history shows that there is a loss of 2.5 years of life for every year of delay in diagnosing the disease. Hence, the *late major* time history has an initial state which could be labeled as being in the local phase with the remaining states being in the local–metastatic phase. On the other hand, for the three remaining time histories, the deficit survival is remarkably constant within each history. This indicates that these three time histories are in the metastatic phase of the disease. The observation of constant deficit survival for these time histories raises the question of the therapeutic value of a radical mastectomy with respect to increasing survival. One may conjecture that a simple mastectomy would achieve the same results. Furthermore, in the *late major* history, all but the state *bd* have no nodal involvement. Hence, a simple mastectomy should achieve the same therapeutic results for all states but *bd*.

TABLE 3.—Summary of deficit survival

History	State	Number in state	Median age (years)	Median survival (years)	Normal median survival (years)	Delay in diagnosis from initial state (years)	Deficit survival (years)
Late major	*abcd*	30	54	25.6	25.3		—0.3
	abd	61	57	15.4	22.5	3	7.1
	ab	21	59	10.5	20.8	5	10.3
	bd	67	59	5.7	20.8	5	15.1
Early major	*acd*	15	46	16.1	32.7		16.6
	ad	34	51	11.8	28.0	5	17.2
	a	23	53	10.5	26.1	7	16.6
	d	48	57	5.2	22.5	11	17.3
Late minor	*bcd*	12	54	7.4	25.3		18.9
	b	55	56	3.4	23.4	2	20.0
Early minor	*cd*	12	46	10.0	32.7		22.7
	U	62	54	2.6	25.3	8	22.7

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Familial Factors Relating to Prognosis of Leukemia and Lymphoma[1, 2, 3]

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ONLY IN recent years has there been a growing realization of the role of genetics and heredity in modifying the human host susceptibility to carcinogenesis. However, relatively little investigation has been given to the significance of familial case aggregation in leukemia and lymphoma, either in terms of the role that environment may play in contributing to an epidemic of slow-virus initiated neoplasia within the family unit, or whether genetic variations in host resistance to neoplastic transformation account for the striking case aggregations of lymphoproliferative neoplasms.

Several reasons contribute to the lack of wide recognition of multiple-case families of leukemia, lymphoma, and myeloma, which were described initially by Jackson and Parker *(1)* and Videbaek *(2)* and later by Dameshek and Gunz *(3)* and Gross *(4)*. Often multiple generations are involved, with decades separating successive cases, so that no reliance can be placed on the usual perfunctory hospital history of familial diseases. Furthermore, the rapidity of death in juvenile cases treated years ago and the nonspecific symptoms undoubtedly have obscured diagnosis before the advent of increased therapeutic interest in leukemia in the mid-1940's. Because of the unusually high prevalence of lymphoid neoplasia in Nebraska, approximately twice that of the national average, some years ago practicing physicians in several communities began to bring to our attention family "clusters" of hematopoietic neoplasms that had been encountered *(5)*.

METHODS

In Central Nebraska, over 85% of the residents reside in the county of their birth, and good medical care has been available for over a half century, with a few pathology groups serving the community hospitals, facilitating confirmation of diagnosis. The reasons for including all hematological neoplasia together have been previously discussed, based on pathologic, etiologic, and diagnostic similarities.

The studies have progressed over a period of 7 years, to a complete incidence study of all cases of leukemia, lymphoma, and myeloma noted in a 6-county area in Central Nebraska around the city of Kearney over an 11-year period, 1958–68, with a population of 107,440. Last summer (1969), with the aid of the Whedon Cancer Detection Foundation of Sheridan, Wyoming, a comparable incidence survey was completed for 7 southwestern Wyoming counties with similar racial background and population (105,822) for the decade of 1959–68, with a major climatic variable resulting from a mean altitude of 6,300 feet, compared to that of Central Nebraska, 1,900 feet *(6)*.

From the Nebraska 6-county study, supplemented by a review of most of the hematological neoplasms referred to the Nebraska Medical Center, we have compiled the largest number (55) of multiple-case families with up to 6 cases of leukemia, lymphoma, and myeloma in one or more generations ever reported. Three-quarters of the multiple-case family data were obtained by a registered nurse interviewer through intensive family interviews in the home. The prognosis of "sporadic" cases of leukemia, lymphoma, and myeloma will be compared to the prognosis of multiple-case families, all originating from the same midwestern environment, rich in hematologic neoplasms in chickens, pigs, and cattle, with whom there has been much human contact during the past 50 years.

Increased susceptibility to leukemia, lymphoma,

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[1] Presented at the Cancer Clinical Investigation Review Committee Symposium, Cascades Conference Center, Williamsburg, Va., February 19–21, 1970.

[2] Sponsored by the National Cancer Institute and the University of Rochester, Rochester, N.Y.

[3] Supported in part by Public Health Service Cancer Control grants to the Nebraska State Department of Health 1963–68.

and myeloma in family aggregations may be shown by an increased proportion of cases developing in the first few decades of life, with or without increased mortality rates. Chronic asymptomatic lymphatic leukemia is far more common among the elderly than in the young, who usually develop the more acute forms of leukemia. Hodgkin's disease, however, tends to have a poorer prognosis after the age of 40, in terms of years of survival, whereas plasma cell neoplasms are almost exclusively a neoplasm of the middle aged and older patients. In our lumping of all forms of leukemia, lymphoma, and myeloma together in the multiple-case family study, prognostic factors specific to each of these neoplasms have been pooled to trace multiple pathologic expressions of what may be a common oncogenic virus. In any interpretation of the data, it must be borne in mind that diverse neoplasms are included in this study.

OBSERVATIONS

Family Relationships in Multiple-Case Families

At the present time, there are 55 multiple-case families which have been documented with varying degrees of success. These cases have included 76 additional neoplasms within these families, related in differing degrees to the proband case that originally called our attention to the family (table 1A). There were two husband-and-wife-combinations and one stepson in these multiple families (4%), indicating a predominant hereditary relationship to the remaining 96%. The genetic aspects of the familial relationship were emphasized by the 34 sibling and parent-child relationships which were encountered, amounting to 44% of a total expected relationship of 50%, for an autosomal genetic trait of intermediate penetrance, which might be found in about one-half of first-degree blood relatives *(7)*. Among the second-degree relatives, either aunt-uncle or niece-nephew, or grandparent-grandson or granddaughter, who might have only a 25% sharing of genes in common, were found 23 cases, or 30% of the total group. Finally, in the more remote relatives, such as cousins, great uncles or great aunts, and grandnieces or grandnephews, 17 cases were found by family survey, or 22% of the total cases. Although the total number of relatives has not been ascertained in the more remote generations from the proband, the population at risk would be much larger for second- and third-degree relatives than for first-degree relatives, emphasizing the centripetal nature of the familial lymphoma relationship.

Comparison of Mortality: Multiple- Versus Single-Case Families

If the age and mortality of the known multiple cases in families are charted against the single-case families collected during a similar period in the same geographic area with similar population, one is immediately impressed by the high mortality and morbidity seen in the patients under age 20 with a familial lymphoma background, compared to sporadic cases encountered among families with only a single proband in the 2 or 3 generations available for study. If the 2 sets of cases are divided into those under 40 and those over 40, members of multiple-case families have a higher mortality rate in both the young and aged (table 2). Before 40 years of age, 36% of the multiple-case patients have died, compared to only 11% of the sporadic single-case patients. After 40, another 40% of multiple-case patients were dead at the time of survey, compared to only 32% of single-case

TABLE 1.—Multiple-case family study in Central Nebraska. Closeness of family relationships, 55 families

		Type of relationship		
	Unrelated	First degree (siblings, parent-child)	Second degree (grandparents, aunts, uncles)	Third degree (cousins, grandnephews, etc.)
A. 55 Nebraska families—to February 1970	3	34	23	17
B. Literature to 1957 *(9)*	2	48	6	5
Family history of Hodgkin's disease in relatives of 440 patients	1	12	3	6
C. Literature since 1957	0	29	3	2
Total multiple-case families=194	6 (3.1%)	123 (63%)	35 (18%)	30 (16%)

patients. A positive family history of lymphoma, leukemia, or myeloma would seem to offer a poorer prognosis for a given patient with one of these groups of neoplasms, with a greater chance for an early death.

A casual search of the literature for reports of similar types of families tends to verify the impression as to the unfavorable prognosis of the multiple-case families (table 3). DeVore and Doan summarized the literature on familial aspects of Hodgkin's disease up to 1957, adding 15 familial cases noted in histories from 440 of their own cases [table 1B *(9)*]. Undoubtedly, the catastrophic nature of the family "epidemic" led to a selection of these cases for publication, but they do resemble those we have collected over a decade in Nebraska, in the relatively high percentage of cases developing in persons under 20 years of age, nearly all with acute or chronic lymphocytic neoplasms, with a high case fatality rate. Summarizing the more recently reported family relationships (table 3), 63% first-degree relatives and 18% second-degree relatives have been reported in 194 multiple-case families.

TABLE 2.—Mortality comparison of single-case versus multiple-case families

	Age group (years)	Alive	Dead	Total	Mortality (%)
109 Single-case families	0–40	14	35*	49	11
	41–80+	103	216*	319	32
55 Multiple-case families	0–40	6	43*	49	36
	41–80+	22	48*	70	40

* Differences significant at $P < 0.001$ by chi square with use of the fourfold table.

Incidence of Other Types of Cancer in Multiple-Case Families

Videbaek was the first to note an increased incidence of other types of neoplasia associated with multiple-case families with leukemia, an observation which has not generally been confirmed. Because of the unusual opportunities for a controlled investigation into the family background of a large number of sporadic and familial hematologic neoplasms in Nebraska and a uniquely large series of detailed family histories obtained by the clinic staff of the Platte Valley Medical Clinic in Kearney during the past 2 decades on a good cross section of the Central Nebraska population visiting the clinic for all types of illness, a study has been completed of the known incidence of carcinoma and other sarcomas in this population. A total of 255 families were interviewed by a registered nurse using a standardized format over a period of several years. The control group was selected by random investigation of every twentieth noncancer record in the Platte Valley Medical Clinic, with subsequent interview of cooperative families, which finally totaled 117. An additional 11 families were selected and interviewed on the basis of a proband with carcinoma of the lung, which was deemed to represent almost purely an exogenously induced carcinoma. Subsequently, an additional 49 lung cancer families were interviewed from other sources than the Platte Valley Medical Clinic in this Central Nebraska area. A similar interview was carried out in 108 single-case families with one proband having had myeloma, leukemia, or some type of lymphoid neoplasm, and 30 multiple-case families had been interviewed by the time table 4 of this data was prepared.

The average size of recorded families varied somewhat, the largest number having been identified in the multiple-case families (possibly related to the vigor of our interest and that of the family in recalling a catastrophic disease situation). However, a large number of relatives were identified among controls, only a few less than in the multiple-case families and more on the average than the families with single cases of leukemia and lymphoma.

A nearly identical percentage of relatives with cancer was recalled by the interviewed family member in the single-case leukemia families as in the bronchogenic carcinoma families, while only two-thirds as much cancer was recalled by the controls interviewed. However, in the multiple-case families, the occurrence of recalled carcinoma was nearly 50% greater than in the single-case families or lung cancer families, suggesting a real concentration of additional neoplasia in these families. The

Table 3.—Multiple-case families with leukemia, lymphoma, reticulum cell sarcoma, and myeloma reported since 1957

Reference	Pathology*†	Stated patient age (years)	Interval between cases (years)	Family relation
De Costello A: Med Klin 35 : 1255, 1939	CLL (6) AL (1)	13, 40+, 45, 48 (2 gen.)	1, 4, 4, 10, 11 (31 total)	Father, aunt, uncle, 3 cousins (all siblings)
Anderson RC: Amer J Dis Child 81 : 313, 1957	ALL (1) CLL (3) CML (1)	4, 5, 6, 8, 8	1, 2, 5, 6	5/8 siblings
Reilly EB, et al: Arch Intern Med (Chicago) 90 : 87, 1952	CLL (3)	36, 54, 56	2, 4	3 siblings
Gunz FW, Dameshek W: JAMA 164 : 1323, 1957	CLL (3)	53, 56, 56	27	Twins, son of 1 twin
Johnson MJE: J Indiana Med Assoc 50 : 206, 1957	AL (3) LS (1)	24, 26, 29, 33	—	4/12 siblings
Smith F: Brit J Cancer 15 : 731, 1961	RCS (2)	2, 3	3	2 siblings
Campbell WAB: Arch Dis Child 37 : 93, 1962	AL (2)	Neonatal	(Twins) 1, 3	3 neonatal siblings
Gordon Australasian Ann Med 12 : 202, 1963	AML (2) AL (2)	2, 29, 47, 58	4, 12, 36	Brother, niece, nephew
Gunz FW: Brit J Med 2 : 1097, 1962	HD, CLL (2)	64, 67	8, 28	2 siblings, father
Kohlmeier K: Mayo Clin Proc 38 : 523, 1963	AML (2)	?, 5	4	Mother, son
Zachau-Christiansen B: Acta Path Microbiol Scand 68 : 343, 1966	RCS (3)	1, 1 (twins)	1, 3	3/4 siblings
Twomey JJ, et al: Blood 29 : 920, 1967	AL (2)	30, 58	1½	Father, son
Rigby PG, et al: Lancet 86 : 379, 1966	LS (3)	3, 3, 13	1, 14	3/5 siblings
Bierman HR, et al: JAMA 186 : 185, 1963	HD, RCS	61, ?	37	Siblings
Gerstley BJS: Cancer Bull 21 : 88, 1969	HD, AL	3½, 75	4	Parent, grandson
Heath CW, Moloney WC: New England J Med 272 : 882–887, 4/29/65	AL (5)	7, 26, 39, 53, 65	29, 21, 8, 2 (60-yr total)	2 parent-son, 2 siblings, stepson
Fraumeni JF Jr, Vogel CL, DeVita VT Jr: Ann Intern Med 71 : 279, 1969	CLL (3)	48, 64, 65	4, 6 (10-yr total)	3/13 siblings (parents were 2d cousins)

* CLL = chronic lymphatic leukemia; AL = acute leukemia, type not specified; ALL = acute lymphatic leukemia; CML = chronic myelogenous leukemia; LS = lymphosarcoma; RCS = reticulum cell sarcoma; AML = acute myelogenous leukemia; HD = Hodgkin's disease.

† Figures in *parentheses* indicate number of cases reported.

significance of this difference is in doubt, however, due to the larger number of traced family members.

In order to verify the pathology reports obtained from family interviews, a random check was performed of the records of 20 control and lymphoma families to verify the stated cause of death with death certificates filed with the Nebraska Department of Public Health. Of a total of 81 stated causes of death. 11 certificates indicated a cause of death other than that attributed by the family interviewee, or 13.6% error. These data tend to support the validity of the causes of death in these interviews, which also were intensively cross-checked from the files of local hospitals and pathology departments in most of the multiple-case families, verifying pathologically the cause of death from cancer in as many relatives as could be traced, so that a real increase in susceptibility to nonlym-

Table 4.—History obtained by family interview of associated neoplasms in single- versus multiple-case families, and in control families (all derived from Platte Valley Medical Clinic referrals)

	Single-case families	Multiple-case families	Cancer of lung	Control group
Total families interviewed	108	30	11	117
Recorded number total relatives traced	1,541	738	138	2,221
Relatives with malignancy	222	165	21	196
Percent with cancer	14.4%	22.4%	15.2%	8.8%
Average size of families recorded	14.4	24	12.5	19.7

phoid neoplasms would seem to be a definite possibility in many of these families.

Prevalence of Multiple-Case Lymphoma Families in an Area of High Endemic Disease

In an effort to ascertain the prevalence of multiple-case families with leukemia and lymphoma in Nebraska, the family history of 109 randomly selected patients with benign disease who visited the Platte Valley Medical Clinic were reviewed for history of lymphoma, as well as an expanded group of 60 cases of primary lung cancer occurring in the 6-county study area, of which 11 cases had been clinic patients. Of the 169 families, 13 (7.7%) reported one case of leukemia, lymphoma, or myeloma in 2,790 traced relatives, and 3 families with multiple cases of disease were found (1.8%). This figure is in contrast to the 3.6% incidence of multiple-case families reported by DeVore and Doan in 440 relatives of patients with Hodgkin's disease *(9)* and 1.3% similar incidence in Jackson and Parker's 237 cases of Hodgkin's disease *(1)* (table 5).

TABLE 5.—Central Nebraska multiple-case family study: Control families obtained by random selection of index case, Platte Valley Medical Clinic, Kearney, 1968

Proband diagnosis	Total No. families	Families with 1 case leukemia and lymphoma	Multiple-case families	Leukemia, lymphoma, or myeloma history (%)
a. Lung cancer	60*	4	1 (3 leukemia)	8.3
b. Benign disease	109	9	2 (2 leukemia RCS & AL)	10.1
Total	169 (2,790 relatives traced)	13	3	9.5

Multiple-case family incidence in this study 1.8%
DeVore and Doan in 440 Hodgkin's disease cases (*9*) 3.6%

* Supplemented by 49 other primary lung cancer families from other sources in the Central Nebraska 6-county study area.

Types of Hematopoietic and Lymphoid Neoplasia in Relatives

The types of disease noted in relatives is summarized in table 6, showing that acute lymphatic leukemia with 21 related cases was the most frequent, although only 8 probands had this diagnosis. The most frequent congruent diagnosis was Hodgkin's disease in relatives of patients with Hodgkin's disease.

The brief lifespan of patients with acute lymphatic leukemia in earlier years and the adult nature of the patient population at the Platte Valley Medical Clinic from which over two-thirds of the probands have been drawn probably account for the predominance of lymphosarcoma, Hodgkin's disease, and chronic lymphocytic leukemia in the probands. The infrequency of acute or chronic myelogenous leukemia is noteworthy among the multiple-case families, as well as among the total patient population obtained over an 11-year period from the 6-county area around Kearney *(5, 8)*. A random survey of well adult individuals obtained from randomly selected households in this region has shown that 25% of adults exceed Wintrobe's normal standards for absolute or relative lymphocytosis, suggesting that the lymphoid system was reacting to some type of lymphoproliferative agent at the time of survey *(11)*.

DISCUSSION

On the basis of the data derived from these three principal sources: *a*) detailed family histories of all leukemia, lymphoma, and myeloma patients, in our own experience and that reported in the literature, and *b*) comparison of these family histories with those of other randomly selected patients with or without carcinoma in the proband, and *c*) comparison of the single sporadic to the multiple-case family with leukemia or lymphoma, we can confirm other reports of familial aggregation of both lymphoid and nonlymphoid neoplasia in our area of maximal endemic lymphoma prevalence. Whether these families represent genetically susceptible "cancer families" such as have been summarized by Lynch *(10)* remains for future research to establish. As a result of the twofold difference in lymphatic leukemia and fourfold difference in lymphoma incidence rates

Table 6.—Disease interrelationships noted in Central Nebraska multiple-case family study (February 1970–55 families)

Diagnosis of proband	Number of probands	Diagnosis most frequent in relatives	Total relatives with disease diagnosis
a. Lymphosarcoma or lymphoma, unspecified	10	Acute lymphatic leukemia	5
b. Hodgkin's disease, giant follicular lymphoma	10	Hodgkin's disease	12
c. Chronic lymphocytic leukemia	9	Acute lymphatic leukemia Chronic lymphocytic leukemia	6
d. Acute lymphatic leukemia	8	Acute lymphatic leukemia	21
e. Acute myelogenous leukemia	4	Acute lymphatic leukemia	2
f. Chronic myelogenous leukemia	4	—	1
g. Multiple myeloma	4	—	2
h. Reticulum cell sarcoma	3	—	0

between Central Nebraska, and the high arid mountain plateau region of southwestern Wyoming observed over a decade in a genetically similar population, we are inclined to believe that the high Nebraska lymphoma incidence may reflect the addition of environmental circumstances. The intense exposure to avian, bovine, and porcine leukoses has been experienced by over 90% of Nebraska residents during the last 50 years. Such exposure has not been possible for the racially and economically comparable residents of Wyoming where climatic severity and aridity have minimized domestic animal contact. Both genetic factors and environmental transmission via milk have been implicated in bovine leukosis, which closely resembles in pathology and natural disease history, human lymphosarcoma and lymphatic leukemia, so that both factors will probably require careful evaluation in future studies *(12)*. Whatever the cause, the prognosis of familial lymphoproliferative neoplasia has a much poorer prognosis than that of the sporadic cases. The combination of genetic and environmental factors underlying the development of neoplastic disease in this available population presents an attractive model for future etiologic investigations.

SUMMARY

In an area of high endemic prevalence of lymphoid neoplasia (Central and Eastern Nebraska), single cases of leukemia (all types), lymphoma (all types), reticulum cell sarcoma, and myeloma have been found in 7.7% of detailed family histories obtained from noncancer patients seeking medical assistance. An additional 1.8% of families report two or more such cases, making a total 9.5% prevalence of chiefly lymphoid neoplasms among families of noncancer patients.

In 55 multiple-case families, which were compared to 109 single-case families similarly studied by a registered nurse interviewer, whose histories were rechecked against available pathologic and clinical records, the case fatality rate was greater and occurred at an earlier age, suggesting especial genetic susceptibility, due to the 74–80% concentration of cases in first- and second-degree relatives. Environmental contribution to the excessive morbidity and mortality in this area is suggested by 50–75% lower case incidence data obtained over a decade from racially similar populations living above 6,000 feet altitude, 1959–1968, in Southwestern Wyoming.

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DISCUSSION

Chairman: Before going into the general discussion, I would like some comments from the co-authors of the papers given at this panel, Dr. Taylor and Dr. Lemon.

Dr. Taylor: I would like to bring up a few points about Dr. Cutler's paper. In some of these cooperative study sessions, our peer may have a regression rate of 52%, while we may have one of 12%. It is not that he is a better clinician or a better liar than we are, but that there has been a definite difference in the selection of patients. I remember a very heated discussion when one individual had a 7% regression rate and he "should have had" an 18.8% regression rate. The thought was: "Well, maybe the clinician doesn't know how to look for a regression." But undoubtedly he had different types of patients. I remember going on a "site" visit in a cooperative study, and the patients had a 58% regression rate, when they "should have had" about a 30% regression rate. Those cases were obviously unusual; it was an estrogen study and the women were all in their 70's. The physician had been studying the use of the drug in a home for aged women. These women had soft-tissue disease, on which estrogens work very nicely. At the same time, cooperating with them was a group in New York that was treating patients who were in thier middle 50's. This group was getting a regression rate of about 7 or 8%.

Dr. Olson: Dr. Taylor presented the problem that we have. I wonder if we have seen two solutions. Is there any way to meld these solutions? How can we get away from the problems of the cooperative groups that Dr. Taylor mentioned by selecting patients by geographical location or by hospital type? Obviously the patients at the Mayo Clinic are different from those at Bellevue. The interesting thing to me was that Dr. Cutler and Dr. Taylor could not get their material from the cooperative groups because it was not available to them and they had to go to separate sources. Is there any way that the accumulated material in the cooperative group can be analyzed in a method similar to Dr. Feinstein's?

Dr. Taylor: I brought this point up for several years at each of the cooperative breast cancer meetings. We got 3 volunteers for this, and some of this material is from cases sent in from the cooperative groups. But we had to go to the source of the material at the institution, because the type of information we needed was not included in the pooled material. That has been the sad situation about the cooperative groups; we have not sent in the important material on these cases. Because we were afraid that we were asking too much information, we got less and less.

Dr. Feinstein: First, with respect to the variations among patients in different hospitals, we have to assume either that some mystical officer assigns different kinds of people to hospitals for random, haphazard, and unknown reasons or that, since these groups could be distinguished by their different clinical properties, if we make a better attempt to classify these properties, we can better classify the patients. For example, to get to the Mayo Clinic, one has to be healthy enough to travel there. Now, patients who have a cancer anatomically of the same type and stage in a municipal hospital, to which they did not have to travel far, may be more likely to be fatigued, to have lost more weight, and to have had a comorbid disease than the patients who were well enough to travel to the Mayo Clinic. If we were then to recognize some of these other clinical properties, in essence to determine how "sick" these people are and make appropriate statistical arrangements for them, we very well might be able to allocate people from different hospitals and to appropriate kinds of categories. Then they really could be compared. For example, in cancer of the lung, the survival rate seems to be better among women than among men. But if you then look at survival rate from a cancer within a comparable clinical anatomic stage, survival rate is the same for women and for men. The reason the overall rate seems higher among women is that more women are discovered while they are asymptomatic, proportionately. Thus your population includes more women in this relatively favorable asymptomatic group. But, if you then look within the asymptomatic group and compare women and men, the survival rate does not seem to be much different. That leads me to answer your second point: Given a variety of different things to look at, how can we avoid having 12 million kinds of cells or categories that we have to contemplate? It seems to me that in essence we look at gradients within gradients. In other words, if we think that there is a gradient for sex, women doing better than men, and if we think that there is a gradient, say for anatomic stage, let us then look at women and men with cancer in the same anatomic stage or in different anatomic stages and see what happens. If we get gradients going in both directions, then presumably both these things are important. If some gradients disappear, then we can recognize that this is not a particularly important property and we can ignore it. Having thus found the critical kinds of variable that we ought to consider, we will still have a great many variables. We will then have to decide how to avoid having myriads of cells. We could cluster the variables on the basis of the groups that have comparable prognostic outcomes. What is a cluster of variables that determines a common prognosis? You can group the variables together by looking both at the initial state and at the subsequent state, and you can thus develop your categories.

Dr. Lemon: Dr. Feinstein, have you left out something extremely important, the interest and the enthusiasm of the physician prescribing the medication? This is particularly true in a disease that can express itself over a period of even one or more decades, such as breast cancer. The interest of the patient or the interest of the physician in the care of his patient can make a difference in the series.

Participant: It seems to me that the other major problems of Dr. Taylor and Dr. Cutler are how do we know what the pre-interval is, how do we know what the sites of metastases are, and how do we know what the menopausal status is? Take metastases: We all like to do brain scans and bone scans to determine sites of metastases, for these scans can help us. Yet, no one has the funds to do these scans, and scans are not practical, so you are feeding into your studies a lot of other variables. Some patients may have had a hysterectomy and, if one assumes that these patients are menopasual, they may not be oophorectomized.

Dr. Taylor: That is terribly important. In our next study, we are putting in what was done to establish the diagnosis of disseminated disease. The people whom we asked to get this material for us were from institutions where we knew careful work was being done. I think that they are fairly comparable from that point of view. Five years ago we were not doing liver scans routinely, and some institutions still cannot do liver scans; so our data on liver metastases are poor. At Roswell Park Memorial Institute, adrenalectomies are being done with very good results on liver metastases because they can reveal liver metastases when they are in the abdomen. So these metastases are found earlier and may not seem to be so bad as they appear to our group.

Dr. Friedell: We must be very careful in extrapolating from some kind of pathological finding to some kind of function. In particular, I am talking about sinus histiocytosis. This is a morphologic entity with a functional, behavioral concern, *i.e.*, immunologic response. It is detrimental to the understanding of this whole business to confuse the two. Another point has to do with Dr. Feinstein's presentation. We all tend to set up a straw man and then knock it down to make a particular point. But anatomic staging comprsies not only measurement of the extent pathologically but also physical examination and so forth, so that you have a spectrum of decisions in this too. Also, I would emphasize Dr. Lemon's point; in the few medical schools that I have known, the departments of medicine seemed to be least concerned about oncology. We should get more people who are concerned about histories, more concerned about oncology.

Dr. Olson: Some years ago, one group worked out a scheme of chemotherapy for Hodgkin's disease and they got fantastically good results. We could not achieve such results. Then one day a patient who originally had been our outpatient moved to the city where the group worked. He needed chemotherapy for stage III disease. So we thought that we should refer him to these people who were doing such superb work. We found out that, by their criteria, he could not be admitted to their studies. The second story has to do with an X-ray conference a couple of months ago where we were looking at the abdominal flat plate of a patient with Hodgkin's disease who was under treatment because he was sick. He had lost weight, had become anemic, was tired, and did not think he could continue to work. The lymphogram revealed involved abdominal nodes. The patient was placed under treatment and responded very well. His appetite returned, his weight increased, and his hemoglobin rose. He was ready to go back to work. We were having this meeting in the X-ray department with the physician in charge of therapeutic protocol. This physician was on his knees begging the radiologist to find unequivocal shrinkage of the nodes because, if the radiologist was not going to report such shrinkage, we could not classify this man as having had an objective response, despite his weight gain and rise in hemoglobin. If you cannot measure a lesion, the only valid objective criteria are fever, pruritus, and cough; this patient had none of these.

Dr. J. Graham: In our series of the cervical cases, we found 3 unsuspected carcinomas of the breast. The patients with these carcinomas died of breast cancer. What do you do if a patient dies after 4 or 5 years and at postmortem has no disease? Do you throw him out of your studies, or do you include him?

Dr. Cutler: What you do depends on who you are. We can handle this by various techniques. If a person dies and evidence (that we would be willing to accept) indicates that he was disease-free, we consider this as a withdrawal from the risk of dying from the disease about which we are concerned, and we can make adjustments.

GENERAL DISCUSSION AND SUMMARY

Chairman: To return to the opening session of cell kinetics and therapeutic responses, Dr. Skipper, do you wish to add anything based on what you have heard or what you have thought since you gave your talk?

Dr. Skipper: One of my favorite philosophic sayings goes something like this: "The facts we use to prove our case convince because we believe already." If I had one thing to say in summing up what I have heard at this fine meeting, it would be that concepts in the long run are going to have to carry over to other disciplines and that our chances for success will be better when we can think in terms of concepts across disciplinary boundaries, such as biochemistry and kinetics (a poor man's biochemistry), genetics, immunology, pathology, pharmacology, subhuman and human clinical responses, surgery and radiotherapy, and chemotherapy and immunotherapy.

Dr. Tannock: I would like to comment on animal tumor models and to make an appeal for using more realistic tumors. Particularly, earlier in this Conference, when people were talking about biochemical pathways, it was evident to me that the actions of drugs depend on the metabolic state of the cells and the metabolic state of the cells depends on the environment of the cells. We heard a lot about drug research done in what I would call artificial tumors, particularly ascites tumors like the L1210. Yet the ascites tumors differ greatly from the tumors with which the cilnician deals. They are avascular, they are anoxic, they have lower concentrations of glucose and many other metabolites, the *p*H is low, and their cell cycle kinetics is totally different from anything we find in solid tumors. For these reasons, we must be very careful about extrapolating from this type of tumor to human tumors. I appeal for the use of slower growing, early transplants of spontaneous animal tumors as a better model system. Dr. Skipper is certainly progressing in this direction, and it would be nice if some of the pharmacology and the biochemistry workers would also do this.

Dr. Hall: One thing that struck me particularly with Dr. Skipper's presentation was his concern about the long-persisting cell that was not perceptible. Perhaps not enough attention has been devoted to the hormones as agents which do not kill cells but take cells out of S and M and put them into G_2 where they are differentiated and live but yet do not grow. The more cells "vegetate" in G_2, the better the condition of the cancer patient. It is very important to keep that fact in mind in kinetics, because often a very large nondividing pathway is ignored. In terms of prediction of response, a neoplastic tissue of an endocrine-sensitive type, such as tissue of breast cancer, which can be put into an organ culture, treated with a hormone, and become more differentiated, *i.e.,* make casein, may need to be treated with estrogen *in vivo* so that it can leave S, go to G_2, and cause a regression in the patient. A cell permanently in G_2 or G_0 is as good for the patient as a dead cell.

Dr. Mihich: I agree fully with what Dr. Tannock said. However, I think that, concurrent with this awareness of the model systems which are slow growing, we should understand their limitations and ask only these questions that the model can provide answers for. We would not have many clinically effective anticancer drugs today if we had limited our testing to a few models. I think that many models are useful, provided that we know what we can get out of them and know their limitations.

Dr. Regelson: If we are going to deal with models, we ought to look at models that are not necessarily malignant. For example, why does the tropoblast survive? It may metastasize and then regress with parturition. Why does a placenta survive? Why does the fetus survive? It strikes me as representing a turning-off of cellular immunity at a very striking focus. Again you have the miracle of the ovum—God bless it! After 40 years of remaining quiescent, it then goes into reduction division. What turns it on? If we set our sights on models associated with the developing embryo in the uterus and with the turning-on or turning-off of the egg or the endometrium, then we may come up with not only an anticancer agent but also an antifertility drug.

Dr. Skipper: One thing that impressed me very much with the mammary tumors with which we work (the first-passage C3H and the 7,12-dimethylbenz[*a*]anthracene-induced) tumors was that they are not "transplanted tumors" at all, but rather are "hormone-dependent" tumors. These tumors grow and have a thymidine index of about 8 or 10%. If one ovariectomizes animals with these tumors, within several days the thymidine index drops from 10, not to 1, but to a tenth of a percent: 100-fold. Then very quickly, if one treats the animals with estrogen shortly after the thymidine index has dropped, the thymidine index will go up again.

Dr. Roberts: On this question of model system, there is one point that should be brought up to the clinician: The human race is made up of a number of compartments and there may be some problem that we deal with in the measured compartment, which is in general the white compartment of the patients. There are more white compartments and therefore more of an opportunity to test their responses to drugs. There seems to be more evidence that the black patient may not respond to these drugs with the same sensitivity as the white.

Dr. Mihich: I was going to raise another variable. This applies to the model system in the 7,12-dimethylbenz[*a*]-anthracene-induced tumor, in the human host, and also to clinical trials of various drugs. A very interesting hepatic microsomal system, the drug-metabolizing enzyme system, can be turned on by very small doses of phenobarbital, phenothiazine drugs, polynuclear hydrocarbon carcinogens, estrogens, or androgens. This is one of the uncontrolled variables. Anyone doing clinical studies should look very carefully at what sedatives are being coadministered to the patient, because he might get two entirely different results with estrogen therapy from a group of patients who are receiving the large doses of phenobarbital and those who are not.

Dr. Heidelberger: It is becoming increasingly assumed by people in the carcinogenesis field that the initial carcinogenic event is a somatic mutation, the result of a direct alteration in the structure of DNA. This need not be a valid assumption, for there are many other models that could explain the initial carcinogenic event. This point cannot be considered as established one way or the other. I would plea for an open mind; to me a derepression type is more appealing than the mutational one, but this important theoretical point is still open.

Chairman: This point is very important. This mutational model has been used in one form or another for very nearly one-half a century. It has been a remarkably unsterile model in terms of getting work done or as a model that could be tested to predict events that occur in the real world. Very little attention is being paid to the impact of the toxic agents, radiation or chemicals, on the normal cell which has to survive all these events if one is going to have a worthwhile therapeutic response. We urge our colleagues in the laboratory to look at what is going on in terms of toxicity in the normal cell, probably first alone and then in growing composite cultures. The second remark I will make probably arises out of my naiveté. Practically everything said about kinetic behavior related to trying to get at the cell in active metabolism. I wonder if we might invert this point a bit and encourage some people to look at the factors that throw cells into G–0, or a nonproliferating stage. As far as I can determine, and I have asked a few of you here, only one person could cite even a smidgen of work going on in this area. After all, having a neoplastic cell would not be so bad if it were not growing.

Dr. Pearlman: Until recently, I have been for years in the Department of Surgery. I still do not understand why adrenalectomy should be beneficial to the patient. We have not had an opportunity to ask this question here. I plea for improved communication between the surgeon and the biochemist working in the laboratory. Fundamental laboratory information can be fed back to the clinician unobstrusively. Thus we might find some correlations between the information that could be of value to the clinician in predicting for the real situation confronting him in the clinic.

Dr. Bulbrook: In regard to Dr. Skipper's remark about cross-disciplines, I am not flogging the steroids because I am a steroid man. But steroids do impinge an awful lot on the data here. For example, one could split Dr. Cutler's survival curve depending on steroid factors. One could sort out much more precisely the epidemiological approach in breast cancer families if one looked at unaffected sisters of cancer patients who also have very peculiar hormone patterns. The hormone environment influences response to immunological agents.

I have been tightening up some general laws from the results we have heard. The first law states that already successful predictions are based on retrospective surveys. The second law states that, once the predictor is successful in a retrospective survey, the investigator drops it like a hot brick. For example, Dr. Nathanson's very elegant work on ^{32}P has been in print for 10 years. It has been picked up, dropped, picked up, and dropped; nobody has ever used it as a predictor. The third law states that, however good a retrospective study looks, no practicing clinician would use it. He will continue to treat his patients in the way his father did. The fourth law states that the rare clinician who tries to use the predictors that we have all been talking about will find that they come apart in his hands. So I think that I should break the news to you that we are all at the wrong conference by some awful administrative error. The real conference is going on in the Californian desert, and we all turned up at the meeting by the relation between biochemical, clinical, and pathological variables and responsive treatment—not on prediction. To use horse-racing terms, anyone can retrospectively pick out which horse is going to win a race but not prospectively, and that is what predictions are about—what is going to happen. All we have here is what has happened in the past. Now in this Californian desert, they have 3 simple race processes: 1) They look for a predictor, as we have all been doing; 2) they test the predictor prospectively; and 3) then they apply the predictor and, if they are lucky, their response rate goes up. There is not a single piece of documented evidence anywhere in the world that, when a predictor has been used and patients have not been treated because they are shown to be insensitive to a particular agent, the response of the remaining patients increases significantly—as it has to if the predictor has any meaning.

Dr. Zelen: When we use the term "early," it may not be early enough. When I talked about the "initial clinical stage," this stage really might be a very late one in the disease, and the individual in that stage who is diagnosed may have had the disease for a long time. In the last part of my discussion, I attempted to show that what we call breast cancer might have several different natural histories. These cancers may be hormone treated, as I am sure they are, but the four paths separated into two pairs, with the initial state being in one patient who was 46 years old and the initial state being in another patient who was 54 years old. Age 46 is in the middle of the period when most women are going through the menopause, and age 54 is essentially post menopausal. So it is clearly an expected phenomenon. With regard to the Guernsey study, what Dr. Bulbrook and his colleagues have shown is that in a prospective study there are hormone abnormalities among the subgroup of the population and, if one follows them, the incidence of breast cancer for those having the abnormalities increases over that of the normal population. This might indicate one has a high-risk group worth studying and following. My own view is that none of these things are related: the biochemistry, the natural history, the biology, and the histology.

Dr. Hilf: We have been using this term "model," and we have not defined it very well. I would like to plead for better definitions. Also, the beauty of a model is the fact that it can be manipulated very well. To find out whether it truly is a good model, we need the support of our clinical counterparts so that we can follow the alterations about which we have been talking in our experimental models in the patient.

Dr. Braunsberg: I plea for much more fundamental work in the action of steroids. Although such work is being done by Dr. Bulbrook, it is still really empirical. We do not know what these androgens are doing nor what estrogens are doing. We still do not know what endrocrine surgery does. Thus none of this steriod work is really being successful in prediction. The future is looking brighter now, for Dr. Jensen's and Dr. Dao's work is indeed very interesting. You must remember that 50% are going to be right anyway; so, when they predict failures, you know 5 of 10 are going to be right. Dr. Dao did not have enough information on the primary tumor to substantiate this, and Dr. Jensen failed in his predictions. Statisticians can very easily tell us whether the number of correct predictions are in fact significant.

Dr. Sokal: I would remind Dr. Bulbrook that we have not all presented retrospective data. Some of us have presented prospective data. Dr. Kondo presented some survival curves of prospective data on groups, using his particular test, and showed better survival associated with these tests.

Dr. Mihich: We now have a number of drugs which are very effective but not very specific. We are trying to increase the specificity of treatment with these nonspecific drugs in various ways. On the other hand, we now know that the host's response to the tumor is very specific but very inefficient. So the question is: Can we combine the two? That is what we are trying to propose. I think that it is a likely proposition. Since we are in the mood of pleas, I would like to make two pleas. The first plea is to the toxicologist that indeed he should add a dimension to the preclinical cytological study of new drugs by assessing the relative antitumor immunosuppressive action of such drugs under various conditions. The second plea is to the therapist that he should try to study combination possibilities based on the sequential exploitation of drugs and immunity. Let us kill as many tumor cells as we can with our drugs first but preserve immunity. If we cannot do this, let us try, specifically or nonspecifically, to increase it later on and see what we can do with the host after our maximally possible, drug-induced cell kill.

Dr. Sokal: After having talked for several days about this, I wonder if immunology will not be the religion of the future. All we have really shown so far is that in certain restricted situations immunologic tests do have predictive value. We do not know yet which immunologic test is best. I caution you to remember that some of the enthusiastic things that the new religion is saying are all hypotheses. We do not really know whether or not we will be able to influence immunotherapy.

Chairman: I will take the prerogative of the chair to point out that immunotherapy is not a new thing. If one goes back to the 1920's, one will find papers published on this subject. Also, drugs are not the only things that can alter the immune mechanisms. Radiation can. Dr. Tolliver showed that there was a biphasic response to total-body radiation with relatively modest doses of 50—150 R. Total-body radiation was followed by an enhanced immune response to bacteria. If the dose was increased, the magnitude of the response was decreased. Dr. Mihich, this may be a system whereby you could test your assumption with respect to the immune component in your Mooney strain. If the immune notion is correct and if the Tolliver data have any bearing, one ought to see an increase in survival in experimental animals after modest doses of radiation before the injection of the inoculant.

My final point is to call your attention to a series of papers by Dr. James Murphy in the *Journal of the Rockefeller Institute* in the 1920's. Dr. Murphy was working with radiation at ranges slightly above the grenz-ray level, and deposition of energy was only a few millimeters into the mouse. Dr. Murphy then could show quite striking changes in the behavior of the host to the tumor post irradiation. There was abundant lymphocytic infiltration in the transplants. This could well have been an instance of enhanced immune response. Those of you who are in this field might wish to review these papers to see if they stimulate ideas that could be examined with the more elegant techniques now available.

Dr. Mihich: Your reference to Dr. Tolliver's work has a counterpart in many anticancer drugs. It has been shown that the immune response is increased if the drug is given at the proper dose and at the proper times in relation to the appropriate amounts of antigens.

Dr. Roberts: The group I would like to scold is not here. That group consists of biochemists and pharmacologists who work with an experimental system, obtain results, and then drop the system before testing it in a human. My clinical colleagues should ride herd on these people. Perhaps if the pharmacologist and biochemist are provided with human material, they will be encouraged to carry their results to a human study. There is evidence from my laboratory, as well as from others, that the metabolic pattern of the cell can be changed by the first drug you give. I encourage the clinician to think of what happens to the cell when the dosage schedule is changed, *i.e.,* when the times are scattered at which he gives different drugs used in combinations. The statisticians have really frightened me, because the number of specimens they suggest that I examine is overwhelming. It will be necessary to think of some way of perhaps cutting down on the number of specimens needed to set up predictive inderes. The work of Dr. Fischer also is discouraging, because we have to deal with the population of cells from most biochemical assays available. In acute lymphocytic leukemia, the induction of remission is approaching 100%; our objective here is not response, but cure. How are we going to know what the minority population of cells is like before a relapse occurs?

Chairman: Dr. Lemon, would you like to speculate on the genetic implications of the material that you presented? In particular, how do you define your control groups?

Dr. Lemon: I am not sure there is a satisfactory control group. We tried to go through the same tunnel, the major source of our lymphoma probands, the Platte Valley clinics, where we selected every twentieth patient who came in for complaints other than neoplasia. Into any control group you pick, the geneticists and the statisticians can poke holes. Therefore, perhaps we must look at different populations. Thus we looked at the population in Wyoming, because it was racially very comparable and 85% of the population were living in the country. The data gathered thus far may provide us with a very interesting control that is part of the close association with animals. It has been known for 20 years that the leukemia mortality rates, as reported to the U.S. Office of Vital Statistics, are consideraby less in the Mountain States than in the Plain States in which we are located. An unpublished study in Colorado indicates a sparse occurrence of leukemia. Perhaps a main point is that the people who have done so much work are only looking at a small fraction of the human problem. Are these diseases the same or different? Certainly, in animals, the same virus can give rise to multiple cell expressions, with either leukemic or solid tumor manifestations. This is something we should be aware of in looking at the studies of twins. Much work has gone into the study of leukemia in twins, and the concordance is not as great as many people had hoped. About 10 years ago, a group in the Boston area followed up children born of leukemic mothers and failed to find any leukemia in the offspring over a 10–12 year period, although it is well known that white blood cells do transfer across the placenta into the fetus. There is no question here that there would be ample opportunity for the fetus to become infected, if you will, or parasitized. All we can say, in this particular instance, is that the interval of observation was not sufficiently long for the full expression of the disease. The other main point to make is to back up Dr. Bulbrook: Once you are looking at cancer, you are looking at a very variable, highly mutating cell population, and the biochemist and the chemotherapist are always going to be in a bind trying to figure out what is the best thing to try and do. We must return to the more stable pattern in the preleukemic and precancerous stages and try to identify these stages, for at this point we should have a more stable cell population for studying.

Dr. Feinstein: I want to respond to Dr. Roberts' chagrin about the many patients whom we must examine to try and find out some things. It is a pity, but that is life. What I found more frightening is the oversimplifications made in an effort to avoid coming to grips with the complexities. For years, many of the complexities were avoided because of the problems of dealing with data. There were many things that had to be examined and many people on whom data had to be assembled. It is hard to do this with 3 × 5 index cards and an abacus was difficult to produce. Fortunately, if we use computers wisely, which we do not always do, this problem of handling material may be solved. One main problem will be in getting decent data. Yours was the third appeal that I have heard to clinicians to come to the rescue and try to help. It is really interesting to hear the statement that the clinician has something to offer. For many years, the clinician has been systematically trained to regard his own observations as being worthless, and so he makes them badly. The histories are poorly taken and poorly recorded, just because the clinician has been systematically taught through the years that histories are not really worth doing, that you ought to get the X rays and the laboratory tests, and so on. Thus there are not many clinicians who have been geared to do precise observation, classification, and assemblage of data that we need. If, in the past 40 years, we had done precise assemblage, correlation, and integration of that kind of evidence with other kinds of data, we might have better answers about the fundamental things to which the speakers are now alluding.

Dr. Hall: Genetics is expressed as the genetics of the virus in oncogenesis, or in the tumor once it is established, or in the host. All these factors may affect response to therapy and may give us some hints about predicting responses. For the host, data need to be looked at more carefully in which resistant groups are defined, *e.g.*, Negro children with acute leukemia. In Orientals, there are differences in incidence of breast cancer and of lymphoproliferative malignancies, which may in turn point to something genetic, which is important in our own responses to therapy. It would be a good idea to isolate these presumably genetic factors in the host. In terms of the host again, one thing obviously not well understood is whether a genetic basis to resistance to some drugs is unique to the clone of cells that emerges or whether it exists initially as resistance in the host and the tumor shares this drug resistance with the other cells of the host. If the latter were true, then one would easily predict that selective toxicity, based on the biochemical observation of the drug involved, would be hard to achieve, since biochemical differences would not exist.

With a high-efficiency cloning system, you might be able to predict what Dr. Roberts is asking about. Dr. Knock, Dr. DiPaolo, Dr. Fischer, and others have indicated that, in certain short-range systems, high-efficiency cloning for biochemical variants may be possible. A single viral gene can produce many different tumors, sometimes in the same animal. Are these tumors equally resistant to the same drugs, and is this resistance based on the viral gene or the host? In a human family, when Miller and others report the "broken up tumor syndrome," presumably a virus can enter a family, but a tumor in the thyroid appears in the father, a tumor of the breast in the daughter, and a rhabdomyosarcoma in the grandchild. It might represent a model system in which the mutants characterize each patient's reaction to his environment, or else it could be related primarily to the viral genome and a clinical difference might appear. All patients with neoplasms present within their families should have more similar responses to treatment than patients without a common genetic determinant. Therefore, in a prediction system, we could say that patients with tumors of whatever type in a tumor-family situation should be similarly treated. Have you looked at this?

Dr. Lemon: One situation no one has looked at biochemically. It is at the other end of the bell curve of human-frequency distribution of cancer in families. This is the noncancer family. These families, in which there has been no malignant disease up to 5 or 7 generations, are now documented. The old family Bible has sometimes been very helpful in tracing these families. A determined search should be made for these families so that cytogenetic studies can be done on whether, for instance, their leukocytes, on aging, also develop the chromosomal aberrations that most of the rest of us get each decade as we get older and which result in a larger proportion of cytogenetic changes in our own normal leukocytes. Maybe these noncancer families have a very stable type of DNA resistant to mutation.

On the other question, two factors work in this multiple-family case. Obviously there is more disease in the younger age groups who die sooner. This would be consistent with an overwhelming dose of something or with an inherited susceptibility. At present, all we can say is that there is a great need to look critically at these families from the cytogenetic standpoint. I am unaware of any commonly reported cytogenetic change in lymphocytic neoplasia.

Dr. Kessel: With the current data the art of predictive testing, we do have a pretty good chance for some drugs being effective in some patients. In the future, more tests will be available to predict whether some drugs are ineffective in the appropriate patients. It is well to remember that the predictions work best when whole cells are used.

Dr. Wilmanns: Biochemical predictive tests are not so necessary for the initial remission of induction in highly drug-sensitive tumors such as acute lymphoblastic leukemia. But they can give one a better idea of the best doses and schedules, and, for these, cytokinetic data are also needed.

Dr. Bertino: One can get discouraged in terms of the *in vitro* predictive tests and by seeing the massive data that we have and the difficulties in evaluating clinical responses. However, we should point out the type of study that does lead to some encouragement. That is, if we know the mechanism of action of drugs, if we understand the pharmacology and the biochemistry of the drug, and if we limit ourselves to as homogeneous a population as possible, then this type of approach has some real benefits. I can point out the work of Dr. Wolberg and Dr. Jensen from that point of view. The initial predictive test can predict failures that will help us to at least limit the use of a drug in those patients in whom it definitely will not be useful. More important is the use of biochemical and pharmacological monitoring when we treat patients, by which we can see what the drugs are doing and we can then decide not only when to use the drugs but also more appropriately what dose to use, how long to give the drug, and what drug to be combined with what drug. Kinetics is a process that limits the use of the drug, even though we know the drug will kill *in vitro.* Because we do not understand the growth rate of the cell *in vivo* nor the kinetics of the particular tumors with which we are dealing, the statements we heard in the kinetic area about human tumors are very important. The growth rate and the size of the tumor are very important in the consideration of chemotherapy. If we lump all our patients together clinically, we will find tumors which have plateau phases, in which they are not going to respond to drugs to which they are biochemically sensitive.

Chairman: The last point is a very shrewd one, and more often than not the human patient presents us with a plateau situation rather than the log-phase situation. When testing new agents, one really rationally ought to include that plateau phase as well as the log phase.

Dr. Heidelberger: I have been interested during the last half hour in all the exhortation and admonitions going forth to biochemists and clinicians on what they ought to do. I would save my exhortations and admonitions for my own kind and say: Let us try to keep attempting to make better drugs and let us not neglect the metabolism in man of those drugs that are active.

Dr. LePage: Dr. Heidelberger certainly expressed my sentiments very well. People keep talking about getting "earlier and earlier" tumors. Somebody had data indicating that the average intermitotic time for some of these mammary cancers was 44 days. If you started off with one or a few cells and these cells doubled every 44 days, at what point would you get to a billion cells so that you would have a detectable tumor? It seems to me that you are talking about many years.

Chairman: What you are saying is that this is almost sufficient proof that the doubling time must change.

Dr. LePage: I do not think that the doubling time necessarily does. The doubling time will change, and there will be periods when it will stop. But certainly there are cases where the doubling time is very short, like in the L1210, since obviously some children at 2 years of age turn up with 10^{12} leukemic cells.

Chairman: No one has mentioned yet the data of Dr. Hugh Tomlinson of the Experimental Radiology Research Unit, Medical Research Center, Hammersmith Hospital, DuCane Road, London W 12. His was the only study of which I was aware that tests the implicit assumption (all too frequently made) that, if you know the generation time and add your toxic agent, then this is not going to change it. Dr. Tomlinson has tested this with radiation on a transplantable isogenic line with a generation time of 18 hours. After the first dose of radiation, the generation time was up to at least 64 hours. Here is one concrete instance where the implicit assumption is not satisfied. It seems to me more likely than not, when this is looked at with other agents, this also may occur.

Dr. Wolberg: I would like to comment on what Dr. LePage said. When we are talking about cell generation time, we must also remember that the tumor-doubling time is not necessarily the same and that the tumor's growth is the result of cell division and cell death. I agree with Dr. Roberts that large numbers are required, but that is because we are looking for two small differences. When we get a drug that gives us big differences, we will not have to look for small differences and we will deal with smaller numbers. Another thing is that we are kind of up against it in our predicting systems because it is easier for us to detect clinically normal cell kill when patients get leukopenia than when they get diarrhea. We just cannot detect the same degree of kill with our solid tumors and tissues.

Dr. Sky-Peck: I thoroughly agree with Dr. Bulbrook's observation that prospective studies and predictive tests usually last about as long as it takes to get enough cases about which to publish a paper and then the tests are dropped. Dr. Bertino and Dr. Heidelberger expressed my sentiments on testing, in that one should really test pharmacologically a system about which one has written on biochemically. I am not discounting the validity of the use of a general test. Using a general test, one should not try to get more out of it than what is of value, and one should be aware of its limitations. In the predictive tests, for example, you can predict what is going to happen during the acute phase for a drug during the initial testing period, but so many changes occur within the patient following this that the test does not need to tell you how the patient is going to react over the long haul.

The other thing that I would like to mention is that much has been said about genetics, and I have been doing work on this, similar to that of Dr. Skipper and Dr. Tannock. I do not get the impression that people realize how difficult these tests are, especially in the human. A lot of sampling is necessary because there are things which take place in time. It also depends on what kind of a model you assume for your kinetics. So it is really difficult to use it as a predictive test until we know a lot more about cell biology. In the study of the kinetics of normal tissue, it becomes rather apparent that normal tissues are not that different from tumor tissues and quite often are more active than tumor tissues. Drugs designed only for active metabolism often are self-defeating.

Dr. Heidelberger: Dr. Wolberg has been working hard on this one predictive test for many years. He has been getting some impressive correlations, both prospective and retrospective. The reaction of study section members, many of whom are famous phage geneticists, is: "My, is he still doing this junk? It is unimaginative." Thus grants do not get renewed. So there are penalties to be paid for persistence.

Dr. Dao: Many studies have been obviously repeated and confirmed; furthermore, predictive tests must be simple and be done very easily, especially by ordinary laboratories. For instance, most people are not familiar with steroid chemistry. It is frightening to look into this type of test, and so a lot of people are just afraid to get to first base before they can confirm the results. We have to look at a lot of our problems more in depth before jumping to the conclusion that we can predict. For instance, in our own study, if we had to say that the measurement of sulfa transferase is the answer, we must ask why there is a missing enzyme in this tumor. Since we have 3 reactions and find that it is due to the lack of active enzymes, this opens a field of research, in that we are looking into other aspects, such as steroid sulfate synthesis and steroid transformation by cancer tissues. The clinician frequently feels that all we need to do is to cut a piece of tissue out and send it to the laboratory. The biochemist receives a piece of tissue, does not even know what it is, and starts to process it. Troubles begin when communication is lost. You can get at lot of contamination with normal cells and tissues. If a good assay is to be done, the clinician should go into the laboratory, talk to the chemist, and even do some chemistry; the biochemist should learn something about the clinical aspects of the disease.

Dr. Hall: Out of this Symposium appear to arise a certain number of desirable additions to what is currently being done in the conduct of clinical trials which are under way in the Cooperative Groups:

1) Simple things can be done to improve the data sheets so as to include the epidemiologic data that Dr. Feinstein, Dr. Cutler, and Dr. Taylor would like.

2) Simple immunologic tests such as dinitrochlorobenzene on all patients could be done at essentially no additional effort or cost.

3) In the design of our regimens, we could consult more with those persons who are concerned with drug types and categories so as to include some concepts such as the drug matrix Dr. Valeriote indicated. You do not have to know anything about the biochemistry of the drug except the concept that there may be classes.

4) When other combinations are constructed, they should include various members of the various drug classes.

5) We have learned the kind of things that Dr. Rappaport and Dr. Friedell, our friendly pathologists, do: reviewing and classifying blood vessel invasion and other histologic variables on material that is currently collected by the surgical chemotherapeutic and radiologic groups.

There are other things that Cooperative Groups can learn from this Conference. I hope the CCIRC will write letters at later intervals to see if anything is generated in group behavior from this Conference.

On behalf of the CCIRC, I want to thank all of you who participated as panelists and discussants and all of you who helped arrange the secretarial chores.

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